EXHIBIT 13

United States Patent [19]

Tenold

[11] **4,396,608**

[45] **Aug. 2, 1983**

[54] **INTRAVENOUSLY INJECTABLE IMMUNE SERUM GLOBULIN**

[75] Inventor: **Robert A. Tenold, Benicia, Calif.**

[73] Assignee: **Cutter Laboratories, Berkeley, Calif.**

[21] Appl. No.: **295,916**

[22] Filed: **Aug. 24, 1981**

[51] Int. Cl.³ C08L 89/00

[52] U.S. Cl. ..... 424/177; 424/85; 424/101; 260/112 B

[58] Field of Search ..... 424/85, 101, 86, 87, 424/177; 260/112 B

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,093,606 6/1978 Coval ..... 260/112 B

4,186,192 1/1980 Lundblad 424/85

FOREIGN PATENT DOCUMENTS

47-37529 9/1972 Japan .

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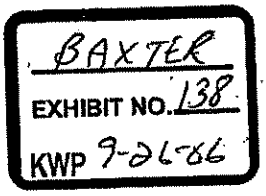
Assistant Examiner—Patricia Short

Attorney, Agent, or Firm—Theodore J. Leitereg

[57] **ABSTRACT**

A composition is disclosed which comprises a solution in a pharmaceutically acceptable carrier of an immune serum globulin, said solution having an ionic strength and a pH to maintain the monomer content and the actual and latent anticomplement activity of the immune serum globulin such that the composition is intravenously injectable. Novel methods are disclosed for preparing the above composition.

14 Claims, No Drawings



INTRAVENOUSLY INJECTABLE IMMUNE SERUM GLOBULIN

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

This invention relates to pharmaceutical compositions comprising novel intravenously injectable immune serum globulin, to a process for its production and to its use to administer immune serum globulin intravenously for human therapy.

Intramuscularly injectable gamma globulin preparations are known. One such product is "HYPER-TET" (Cutter Laboratories, Inc., Berkeley, Calif.).

The usual intramuscular gamma globulin preparations cannot safely be administered intravenously because such administration causes an unacceptably high incidence of reactions, especially in agammaglobulinemic recipients. These reactions have been associated with a decrease in serum complement levels, apparently caused by complement binding by the administered gamma globulin. S. Barandun et al., *Vox Sang.* 7, 157–174 (1962). The ability of gamma globulin to bind complement, termed anticomplementary, is greatly increased as a result of denaturation brought about during the fractionation procedure, in particular by aggregation to high molecular weight species. The complement binding mechanism of these aggregates appears to be identical to that of antigen-antibody complexes. D. M. Marcus, *J. Immunol.* 84, 273–284 (1960). When the aggregates are removed by ultracentrifugation at 100,000 x gravity, a product low in anticomplement activity is obtained which is well tolerated upon intravenous injection. Barandun et al., supra.

Several approaches have been taken to the problem of rendering gamma globulin safe for intravenous administration. All of these are dependent on eliminating its anticomplement activity. Ultracentrifugation (cited above) is technically unfeasible, and the product so derived regains its anticomplement activity upon storage. Treatment of gamma globulin with the enzyme pepsin at pH 4.0 results in proteolytic cleavage of the molecule to give a fragment of about 10,000 molecular weight which has a sedimentation coefficient in the ultracentrifuge of about 5S,A. Nisonoff et al., Science, 132, 1770–1771 (1960). Even though this surviving fragment retains bivalent antibody activity and lacks anticomplement activity and is well tolerated and efficacious in intravenous administration, W. Baumgarten, *Vox Sang.* 13, 84 (1967), the therapeutic effect provided is of unacceptably short duration since it is rapidly excreted, having a circulating half-life of only 18 hours, perhaps somewhat longer in agammaglobulinemic patients, compared to 19.8 days for unmodified gamma globulin. E. Merler et al., *Vox Sang.* 13, 102 (1967); B. Jager, *Arch. Intern. Med.* 119, 60 (1967). Although the much reduced half-life of pepsin treated gamma globulin is probably due in part to the drastic reduction in size of the molecule, there are indications that the rate of catabolism of gamma globulin is related to specific properties of the portion of the molecule digested by pepsin. J. L. Fahey et al., *J. Exper. Med.*, 118, 1845–1868 (1963). This portion of the molecule remains intact in the present invention. An additional disadvantage of the pepsin treatment procedure is that the pepsin which remains present is of animal origin and can stimulate antibody production, particularly upon repeated administration. C. Blatrix et al., *Presse Med.* 77, 635–637 (1969). The use of plasmin of human origin avoids this difficulty and is the basis of a different process for preparation of intravenous gamma globulin.

Treatment of gamma globulin with human plasmin results in cleavage into three components of about 50,000 molecular weight. J. T. Sgouris, *Vox Sang.* 13, 71 (1967). When sufficiently low levels of plasmin are used, only about 15 percent of the molecules are cleaved, with 85 percent remaining as intact gamma globulin. Sgouris, supra. The intact gamma globulin remaining undigested shows little anticomplement activity and has been administered intravenously without adverse reactions. J. Hinman et al., *Vox Sang.* 13, 85 (1967). The material thus prepared appears to retain in vitro and in vivo protective activity. F. K. Fitzpatrick, *Vox Sang*, 13, 85 (1967). One disadvantage of this approach is that the plasmin cannot be completely removed. Thus, degradation continues even when the material is stored at 4° C.

Incubation of gamma globulin at pH 4.0 at 37° C. for various lengths of time has been observed to reduce the anticomplement activity to low levels. It has been suggested that this result may arise from a small quantity of serum enzyme present as an impurity in the gamma globulin. Blatrix et al., supra. As with the plasmin treated gamma globulin, this "pH 4.0 gamma globulin" has been found to regain anticomplement activity, upon storage, at an unpredictable rate, so that it is necessary to assay anticomplement activity before administration to a patient. J. Malgras et al., *Rev. Franc. Trans.*, 13, 173 (1970).

Both plasmin treated gamma globulin, Hinman et al., supra, and pH 4.0 gamma globulin, H. Koblet et al., *Vox Sang.* 13, 93 (1967); J. V. Wells et al., *Austr. Ann. Med.* 18, 271 (1969); Barandun et al., *Monogr. Allergy*, Vol. 9, 39–60 (1975), Barandun et al., *Vox Sang.*, Vol. 7, 157–174 (1962), have shorter half-lives in vivo than unmodified gamma globulin. For example, the half-life in normal patients of pH 4.0 gamma globulin is about 14 days, Koblet et al., supra, while the plasmin treated material shows a half-life of 16 days, Merler et al., supra.

The Centre National de Transfusion Sanguine (C.N.T.S.) in Paris has, by careful fractionation and filtration of gamma globulin from selected fresh plasma, produced an intravenously injectable gamma globulin with low anticomplement activity. Blatrix et al., supra; ibid., *Presse Med.*, 77, 159–161 (1969); M. Steinbuch et al., *Vox Sang.* 13, 103 (1967). It is apparently not totally devoid of anticomplement activity, as it must be administered carefully and reactions do occur in some patients. Cortisone may be given prior to injection to eliminate these reactions, but the apparent incomplete removal of anticomplement activity would seem to be detrimental to its widespread use.

The effects on anticomplement activity of reduction of disulfide linkages of gamma globulin followed by reaction with a blocking agent has been investigated in the prior art. S. Barandun et al., supra, found that treatment of a 7 percent solution of gamma globulin with 0.2 M cysteamine, followed by 0.2 M iodoacetamide, resulted in almost complete loss of anticomplement activity whereas treatment with cysteamine or iodoacetamide alone did not significantly decrease anticomplement activity. Because of the toxicity of iodoacetamide, these investigators did not pursue this approach to an intravenously injectable gamma globulin.

A modified immune serum globulin was described in U.S. Pat. No. 3,903,262. The immune serum globulin was rendered intravenously injectable by first reducing to —SH groups a portion of the disulfide linkages of the molecule and then alkylating the —SH groups. After the product was separated from the reaction mixture, it was sterilized. The so-produced material was intravenously injectable, substantially free from both actual and latent anticomplement activity, having substantially the biological half-life and spectrum of antibody activity of corresponding unmodified immune serum globulin.

Currently, there are several intravenously injectable gamma globulin products available outside the United States. One such product is INTRAGLOBIN of Biotest in Frankfurt. This product is made by beta-propriolactone treatment of gamma globulin (Stephan, *Vox. Sang.*, 1975, Vol. 28, pp. 422–437). The material has a molar concentration of sodium ion of about 0.18 and of chloride of about 0.27. The beta-propiolactone used in its preparation is suspected as a carcinogen.

Another intravenously injectable product is manufactured by Green Cross Corporation of Japan (U.S. Pat. No. 4,168,303). It is a lyophilized, natural gamma globulin preparation having an anticomplementary activity of less than or equal to 20 C'H50 units and 0.06–0.26 parts by weight of a neutral mineral salt such as sodium chloride.

The Swiss Red Cross has an immunoglobulin SCR for intravenous administration. SCR contains more than 80% of monomeric IgG and minor fractions of dimeric, polymeric, and fragmented IgG as well as traces of IgA and IgM. The distribution of IgG subclasses equals that of normal serum. The product is manufactured in lyophilized form and contains 3 g of protein, 5 g of saccharose and a small quantity of sodium chloride per unit. A diluent (100 ml) contains 0.9% sodium chloride.

VENOGLOBULIN (Green Cross Corporation of Japan) is prepared by treating gamma globulin with plasmin. It also contains 0.5 parts of a protein stabilizer (e.g. amino acetate) per 1 part by weight of plasmin treated gamma globulin. The product is distributed as a white powder and is dissolved in a diluent for use. The resulting solution is clear or slightly turbid and has a pH of 6.4–7.4.

An intravenously injectable gamma globulin has been developed by Schwab of Germany and contains 50 mg per ml immunoglobulin, 7 mg/ml glycin, and 7 mg/ml sodium chloride.

Schura of Germany manufactures an intravenously injectable gamma globulin by adsorption onto hydroxyethyl starch. The product is distributed as a solution having a pH of 6.7 and a conductivity of 450 mosm. and containing 2.5% glucose, 165 meq/l of sodium ion and 120 meq/l of chloride ion.

VEINOGLOBULINE is available from Institute Merieux of France. It is a plasmin-treated gamma globulin distributed as a lyophilized powder containing 5 g. of protein and enough glycine and sodium chloride to insure pH and stability. The diluent is 100 ml of water for injection containing 0.9 g. of sodium chloride or isotonic glucose.

U.S. Pat. No. 4,160,763, assigned to Behringwerke AG of Germany, is directed to an immunoglobulin for intravenous administration having reduced complement fixation made by treating an immuno globulin fraction with a low concentration of a sulfitolytic agent and/or phosphate which is sparingly soluble in water. The pH of the material is 7.0, and the product contains 0.85% sodium chloride and 2.5% (g/v) glycine prior to lyophilization.

Teijin Institute of Tokyo is the assignee of record of U.S. Pat. No. 4,059,571 for a novel immunoglobulin derivative. A water soluble composition for intravenous injection which contains the novel derivative is described. The derivative is the S-sulfonated product of cleaved interchain disulfide bonds of gamma globulin.

GLOVENIN, a pepsin-treated human immunoglobulin, is manufactured by Nihon Seigaku of Japan. Typically, a solution of the above product contains 50 mg/ml of pepsin-treated immunoglobulin, 2.25% (w/v) of aminoacetic acid, and 0.85% (w/v) sodium chloride.

Yamanouch Seiyaku is the distributor of GLOBULIN V, a dried pepsin-treated human immunoglobulin (500 mg) containing 225 mg of aminoacetic acid and 85 mg of sodium chloride. For intravenous administration the dried product is disolved in 10 ml of water for injection.

SUMMARY OF THE INVENTION

I have discovered an unmodified intravenously injectable immune serum globulin having an ionic strength and a pH such that the monomer content of the immune serum globulin is greater than about 90% and the actual and latent anticomplement activity is maintained such that the immune serum globulin is intravenously administrable to a broad spectrum of patients.

The product of my invention is prepared by a method wherein an immune serum globulin (ISG) is solubilized to yield a solution of a certain protein concentration. The pH of this solution is adjusted, and the ionic strength of the solution is reduced, to a level such that the monomer content of the ISG is greater than about 90% and the actual and latent anticomplement activity is such that the ISG product is rendered intravenously injectable. The pH and ionic strength are maintained at the above levels during protein concentration adjustment, sterilization, filling into final containers, and the like.

One advantage of the ISG of the invention is that it is intravenously injectable thus avoiding the problems associated with intramuscularly injected material. Furthermore, the present product is substantially free from chemical modification such as occurs in reduction-alkylation, beta-propiolactone treatment, and the like.

An important feature of the product of the invention is that it is substantially free of actual and latent anticomplement activity and also substantially free of polymeric material or "aggregates". Particularly, the product of the invention exhibits enhanced stability over prior art preparations. The material may be kept at room temperature for long periods in the absence of additives with retention of its monomer content and lack of actual and latent anticomplement activity.

Another advantage of the invention is that the intravenously injectable ISG is virtually unchanged in physical measurements and biological functions. Thus, the antibody titers in the present material are not significantly different from the starting material.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The starting material for the process of this invention is unmodified human immune serum globulin. In the specification and claims the term "immune serum globulin" is used to define the substance also referred to in

the literature variously as gamma globulin, IgG and immunoglobulin G. It consists predominantly and preferably of at least about 85 percent of the 7S species of gamma globulin, which has a molecular weight of about 160,000. Any remainder is preferably 9S species, with a molecular weight of about 300,000. Both standard immune and hyperimmune serum globulins, e.g., tetanus, rabies and hepatitis immune serum globulins, can be employed, the modified product being immune and hyperimmune ISG, respectively. Thus, a suitable starting material for the process of this invention is Cohn's Fraction II or Fraction III filtrate. See Cohn et al., *J. Am. Chem. Soc.* 68, 459 (1946); Oncley et al., ibid., 71, 541 (1949).

Fraction II, by ultracentrifugation studies, is predominantly (about 85 percent) the 7S (sedimentation constant of 7) species of gamma globulin with an average molecular weight of 160,000. The remaining protein is essentially 9S material with a M.W. of about 300,000. Wet Fraction II paste (approximately 30 percent solids) is commonly lyophilized to obtain dry ISG powder which is then dissolved and prepared for intramuscular injection as a 16.5 percent sterile solution. Either the wet Fraction II paste or the dry ISG powder is a suitable starting material for the process of this invention.

Gamma globulin obtained by any process which has essentially the same composition of protein components as found in the Cohn Fraction II or Fraction III filtrate can be used as starting material in the present process.

Both standard immune serum globulin and hyperimmune serum globulin can be employed as starting materials. As is well known, the latter is produced from plasma or serum obtained from selected donors who have much higher titers for a specific antibody than is normally found in the average population. These donors have either been recently immunized with a particular vaccine or else they have recently recovered from an infection or disease. These high titer sera or plasmas are pooled and subjected to the usual Cohn fractionation procedures up to the point of isolating Fraction II. The Bureau of Biologics (BoB) antibody standards for hyperimmune serum globulins presently are based on products to be given intramuscularly. These standards are based on the assumption a standard intramuscular dose of the reconstituted globulin (1–10 ml) will be administered. Because the amount of antibody required to achieve a desired immunological response is substantially less when administered intravenously, it will be apparent the I.V. dose will be substantially less than the I.M. dose which will produce the same serum antibody titer. Thus, the dose of intramuscular ISG and hyperimmune serum globulin must be higher than that required to achieve the same serum antibody titer when globulin of the same antibody activity is administered intravenously.

The starting wet paste or lyophilized powder is dissolved in a volume of water or other physiologically-acceptable carrier to provide a protein solution of a concentration of about 0.5–20% preferably about 5 percent. If Fraction III filtrate is employed, the aqueous solution must be concentrated by conventional techniques to the desired protein concentration. Any protein concentration may be used in this method; however, the above-recited range is preferred from a practical standpoint.

After the protein has been dissolved or concentrated, the solution is adjusted to a pH of about 3.5 to 5.0 preferably about 3.8 to 4.2, by addition of a physiologically-acceptable acid such as hydrochloric acid. In general, the pH is adjusted to a point whereat the monomeric material in the protein solution is maintained at a maximum. However, the pH must not be so low as to result in gelation. The temperature should not be harmful to the ISG material. Good results are obtained within the temperature range of about 0°–20° C. It is not necessary to hold the so-adjusted material for any period of time prior to the next step; however, the material may be held, if desired, without detrimental effects.

Following pH adjustment the protein solution is treated to reduce its ionic strength to a level at which the monomer content of the ISG preparation is greater than about 90%, preferably greater than about 95%, and more preferably greater than about 98%, and the actual and latent anticomplement activity is such that the ISG preparation is intravenously injectable. For this purpose the actual anticomplement activity should be greater than about 2 mg protein/C'H50 unit. The non-specific complement binding capacity of the product is determined using optionally titered complement and hemolysin. The complement binding capacity, known as anticomplement activity, is reported as mg protein product capable of inactivating (binding) one C'H50 unit. One C'H50 unit is defined as the amount of protein capable of inactivating 50% of complement in an optionally titered complement and hemolysin system.

The ionic strength ($\Gamma/2$) of the solution should be such that the product as a 5% protein solution has a nephelometric reading less than about 15 NTU (National Turbidity Units), preferably less than about 2 NTU. The ionic strength ($\Gamma/2$) is defined as follows:

$$\Gamma/2 = \frac{\Sigma\{[C^+](Z^+)^2 + [C^-](Z^-)^2\}}{2}$$

where

C^+=cations including metal ions such as Na^+, K^+, Ca^{+2}, Mg^{+2}, and the like,

C^-=anions including halide ions such as Cl^-, Br^-, carboxylic acid salt ions such as acetate or citrate ions, and the like,

Z^+=the charge of C^+, and

Z^-=the charge of C^-.

Preferably, the ionic strength, as defined, is less than about 0.001. The above treatment may be effected by standard procedures such as ultrafiltration, diafiltration, dialysis, etc., or combinations thereof. For example, the protein solution at the appropriate pH may be diafiltered with at least five volume exchanges of water, usually about 4–8 volume exchanges, to reduce the ionic strength to at least about 0.001. During this treatment the concentration of peptides and other impurities such as alcohol are also reduced, generally to trace amounts.

After or during the above treatment, the pH is measured and maintained within the range of about 3.5–5.0.

The protein concentration of the so-treated material is next adjusted to the level desired in the final product, such as, for example, 5%, 10%, 15%, and so forth. This adjustment is accomplished by conventional techniques not detrimental to ISG, e.g., ultrafiltration, reverse osmosis, sublimation, evaporation, etc. Again, the pH of the preparation is maintained within the range of about 3.5–5.0, preferably about 3.8–4.2.

Next, the ISG preparation is treated to render it tonic, i.e., to render it compatible with physiological conditions or render it physiologically acceptable upon

injection. In this respect it is important to note that tonicity must be obtained without raising the ionic strength (as defined above) of the preparation. This end is achieved by adding to the ISG preparation an amount of an amino acid, such as glycine and the like, or a carbohydrate, such as maltose, dextrose, fructose, and the like, or a sugar alcohol such as mannitol, sorbitol, etc., or mixtures thereof sufficient to achieve tonicity. Thus, for example the ISG preparation may be mixed with about 10% maltose (on a weight to volume basis) to render the preparation tonic.

After the above adjustment the product is sterilized, usually by sterile filtration through appropriate media, and then filled into final containers. It is also possible to lyophilize the sterile ISG product after filling into final containers. For I.V. use the lyophilized material is dissolved in medically-acceptable water prior to injection. If the product has not been made tonic prior to lyophilization, the lyophilized material must be dissolved in a diluent containing medically-acceptable water and one of the aforementioned substances in an amount to render the preparation tonic.

The ISG of this invention is primarily intended for intravenous administration although the ISG preparation may also be administered intramuscularly if it contains the appropriate excipients. The composition aspect of this invention therefore relates to pharmaceutical compositions comprising a solution, in a pharmaceutically acceptable aqueous carrier adapted for intravenous administration, of an intravenously injectable ISG of this invention. The ISG is substantially pure. The ISG is present in these solutions in any concentration, either suitable for immediate I.V. administration or after dilution, e.g., with water or diluent as mentioned above, to acceptable levels, e.g., about 1–18 percent solution, preferably about 1–15 percent and more preferably about 10 percent for immediate administration, and about 16 percent for dilution prior to administration. The ISG can be administered intravenously alone or in combination with or in conjunction with other blood products, e.g., whole blood, plasma, Plasma Protein Fraction, fibrinogen, clotting factors such as Factor VIII, Factor IX concentrate, and so forth, and albumin.

In its method of use aspect, this invention relates to the intravenous administration, usually to humans, of a pharmaceutical composition as defined above. The composition is administered in a conventional manner, e.g., in an amount which provides adequate therapeutic amounts of antibody. For a 16.5 percent protein solution, about 1–25 ml is the customary single dose. Administration of subsequent dosages is usually within 1–3 weeks, depending upon the severity of the illness and the time of exposure thereto.

As mentioned above the products of the invention may be incorporated into pharmaceutical preparations, which may be used for therapeutic purposes. However, the term "pharmaceutical preparation" is intended in a broader sense herein to include preparations containing a composition in accordance with this invention used not only for therapeutic purposes, but also for diagnostic and reagent purposes as known in the art; for tissue culture wherein organisms such as viruses for the production of vaccines, interferon, and the like, are grown on plasma or on plasma fractions, e.g., Cohn Effluent II+III, Cohn Fraction IV, Cohn Fraction V, and so forth; etc. The pharmaceutical preparation intended for therapeutic use should contain a therapeutic amount of the present composition, i.e., that amount necessary for preventative or curative health measures. If the pharmaceutical preparation is to be employed as a diagnostic or a reagent, then it should contain diagnostic or reagent amounts of such composition. Similarly, when used in tissue culture or a culture medium the medium should contain an amount of such composition sufficient to obtain the desired growth.

The gamma globulin of this invention is substantially free from anticomplement activity, both immediate and latent.

Antibody titer is not significantly different from the starting unmodified gamma globulin, i.e., it is normal or hyperimmune, e.g., tetanus or rabies hyperimmune globulin, depending on the antibody titer of the starting ISG. The antibody molecules are bivalent, as indicated by their ability to precipitate with antigen.

Another characterizing feature of the ISG of this invention is its absence of proteolytic activity. It is known that some samples of ISG form fragments when stored. Such fragmentation is due to proteolytic digestion by a contaminating enzyme often presumed to be plasmin. Fragmentation is undesirable since it causes a decrease in the amount of active antibody in solution. The process of this invention sharply decreases the proteolytic activity in ISG to undetectable levels or at most to trace levels.

A primary and important characteristic of the present product is its stability. The product may be stored for extended periods of time without significant, if any, change in its antibody activity, monomer content, clarity, lack of anticomplement activity and so forth. For example, sterile, final container material prepared in accordance with this invention has been stored at room temperature on the shelf for greater than 6 months without significant changes in the above-mentioned qualities. This stability is obtained through pH and ionic strength adjustments as described above. The art heretofore has not recognized the relationship between pH and ionic strength on the one hand and intravenous injection on the other. As mentioned above, treatment of gamma globulin at pH 4 is known. However, the so-treated material was then returned to about pH 7 for administration to patients. Furthermore, addition of salts such as sodium chloride was employed to obtain tonicity.

A related benefit of the product of the present invention is its lack of buffer capacity. The present product is surprisingly administrable at pH 3.5–5.0. However, since the ionic strength has been reduced to a very low level, there is very little disruption, if any, of the physiological pH such as that which would occur with the administration of a material essentially buffered at pH 3.5–5.0 by the presence of salts.

EXAMPLES

The invention is demonstrated further by the following illustrative examples.

EXAMPLE 1

The pH of Fraction III filtrate (2100 l.) from the Cohn fractionation scheme (Cohn et al, supra) was adjusted to 4.0 by addition of 1 N HCl. Approximately 40 l. of HCl was added at a rate of less than one liter per minute with thorough mixing. The Fraction III filtrate was then metered into an ultrafiltration system. Ultrafiltration and diafiltration were used to reduce the alcohol concentration as rapidly as possible while holding the product temperature less than 10° C. Cold distilled

water was used to maintain a constant volume of approximately 350 liters. Flux rates as high as 20 l. per minute were observed. When all the Fraction III filtrate had been concentrated to about 5% protein and the product alcohol concentration had been reduced to less than 8%, seven volume exchanges were performed using cold distilled water. The product temperature was permitted to drift as high as 20° C. The immune serum globulin solution was then concentrated to 8% protein and drained from the ultrafiltration system; 120 l. of 8% immune serum globulin was recovered in a clear "water-like" state. This material had an ionic strength of 0.001 (as determined by calculation) and a pH of 4.2. An aliquot of this material was made tonic with 10% maltose at 5% protein. This was filled into 250 ml bottles (60) for stability and other testing. Initial high pressure liquid chromatography (HPLC) results indicated a monomer level greater than 99%. This lot passed all typical testing for IGIV (Table 1). Several containers were stored at room temperature and after six months, HPLC results indicate the monomer content was still greater than 99%.

TABLE 1

HPLC Monomer (99.1%) Dimer (0.9%) Trimer (0) Void (0)	
Anticomplement Activity	3 mg protein per CH50 unit
PKA	11% of reference
Buffer Capacity	16.24 meq./L
Ultra-centrifuge	6.6S 90.8% 9.8S 9.2%
Nephelometer	1.5 NTU

A similar aliquot was made tonic by addition of glycine to a concentration of 0.2 M.

EXAMPLE 2

An aliquot (6 l.) of the 120 l. of 8% immune serum globulin prepared in Example 1 was treated with 1 N HCl to obtain a pH of 4.0 and lyophilized.

Water for injection was added to this material to obtain a 5% protein concentration. The reconstituted material exhibited the following characteristics:

TABLE 2

HPLC Monomer (98.5%) Dimer (1.5%) Trimer (0) Void (0)	
Anticomplement Activity	3 mg protein per CH50 unit

What is claimed is:

1. A stable, sterile, intravenously injectable pharmaceutical composition comprising an aqueous solution of a therapeutic amount of an immune serum globulin, said solution having an ionic strength such that the solution at 5% protein concentration has a nephelometric reading less than 15 NTU, a pH of about 3.5–5.0, and a physiologically-acceptable tonicity.

2. The composition of claim 1 which further includes other blood products.

3. The composition of claim 1 which includes a material selected from the group consisting of carbohydrates, sugar alcohols, and amino acids in an amount sufficient to render physiologically-acceptable tonicity to the solution.

4. The composition of claim 3 wherein the carbohydrate is maltose.

5. The composition of claim 3 wherein the amino acid is glycine.

6. A method for treating immune serum globulin which comprises

(a) forming an aqueous solution of an immune serum globulin,

(b) adjusting the pH of said solution to about 3.5–5.0 by addition of a physiologically acceptable acid,

(c) treating the solution to reduce its ionic strength (Γ/2) while maintaining the pH of said solution at 5% protein concentration has a nephelometric reading less than 15 NTU, and

(d) adjusting the tonicity of the solution to a physiologically acceptable level by addition of an agent selected from the group consisting of amino acids, carbohydrates, and sugar alcohols.

7. The method of claim 6 wherein the solution in Step a has a protein concentration of about 0.5–20% by weight.

8. The method of claim 6 wherein the pH is adjusted to about 3.8–4.2 in Step b and maintained thereat in Step c.

9. The method of claim 6 wherein the solution is diafiltered in Step c.

10. The method of claim 6 which further includes the step of

(e) sterilizing the solution.

11. A composition comprising the product of claim 6.

12. The method of claim 6 which further includes the step of lyophilizing the solution of Step c.

13. A composition comprising the product of claim 12.

14. A dry composition comprising immune serum globulin which upon solution in water has a pH of about 3.5–5.0 and an ionic strength (Γ/2) such that the solution at 5% protein concentration has a nephelometric reading less than about 15 NTU.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 4,396,608
DATED : August 2, 1983
INVENTOR(S) : Robert A. Tenold

It is certified that error appears in the above-identified patent and that said Letters Patent are hereby corrected as shown below:

Column 10, claim 6, lines 21 - 24, correct step (c) to recite as follows:

--(c) treating the solution to reduce its ionic strength (Γ/2) while maintaining the pH of said solution at about 3.5 - 5.0, to a level such that the solution at 5% protein concentration has a nephelometric reading less than 15 NTU, and--.

Signed and Sealed this

Sixth Day of November 1984

[SEAL]

Attest:

GERALD J. MOSSINGHOFF

Attesting Officer

Commissioner of Patents and Trademarks

EXHIBIT 14

— Immunochemical quan-
on. *Immunochemistry*, 2,

IDSELL R. and TALMAGE
ition as a measure of se-

determination of 6,6 S
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Revue Française de Transfusion. T. XIII. N° 2. — 1970

NOTES ORIGINALES

Mesure de l'activité anti-complémentaire des préparations de gamma-globulines injectables par voie intra-veineuse

par J. MALGRAS, G. HAUPTMANN, J.-J. ZORN et R. WAITZ

Centre de Transfusion Sanguine et
Faculté de Médecine de Strasbourg.

L'INJECTION intra-veineuse de préparations de γ-globulines isolées par les techniques de fractionnement courantes peut provoquer chez l'homme des réactions d'intolérance sérieuses. Ces réactions ont été attribuées à l'activité anti-complémentaire d'agrégats de γ-globulines (de 9 S à 40 S) contenus dans ces préparations.

Plusieurs méthodes ont été préconisées pour obtenir des préparations de γ-globulines bien tolérées par voie intra-veineuse :

— digestion pepsinique [9],

— incubation à p*H* 4 et à + 37°C en présence d'une faible quantité de pepsine [3],

— précautions particulières prises au cours du fractionnement et absorption sur charbon actif [1, 4].

Dans tous les cas on obtient une diminution du pouvoir anti-complémentaire des γ-globulines et le parallélisme entre la bonne tolérance clinique des γ-globulines et la réduction de l'activité anti-complémentaire de ces préparations rend la recherche de cette activité indispensable.

La mesure de cette activité anti-complémentaire est en général effectuée selon les indications données par FROMMHAGEN et FUDEN-

Manuscrit reçu le 16-2-70.

BERG [5] mais aucune méthode détaillée, directement adaptée au contrôle des γ-globulines intra-veineuses n'a encore été publiée. C'est pourquoi nous proposons une technique mise au point et utilisée au Centre de Transfusion Sanguine de Strasbourg : elle permet de mesurer avec précision la quantité de protéines de la préparation (en mg) requise pour inhiber totalement 2 unités de complément de cobaye donnant 50 % d'hémolyse (2 unités C' H 50). La quantité de γ-globulines inhibant totalement 2 unités C' H 50 doit être supérieure à 10 mg. (Décret du 11 septembre 1969).

I. - PRÉPARATION DES γ-GLOBULINES INTRAVEINEUSES

Nous avons adopté la technique proposée par BARANDUN et coll. [3]. A partir de la poudre de γ-globulines standard isolée selon la technique de COHN modifiée par NITSCHMANN, nous préparons une solution à 13 % de γ-globulines, le solvant étant l'eau distillée additionnée de glucose (2 %), de glycocolle (1,2 %) et de merthiolate (0,01 %). Après filtration clarifiante, le *p*H est ajusté à 4,0 avec l'acide chlorhydrique N/10 ; on ajoute de la pepsine (1) à raison de 0,01 % du poids de γ-globulines contenues dans la solution.

TABLEAU I

Tube N°	1	2	3	4	5	6	7
Tampon Veronal	—	0,1	0,2	0,3	0,4	0,45	0,46
Gammaglobulines à 5 %	0,5	0,4	0,3	0,2	0,1	0,05	0,04
Complément de cobaye (2 U C'H 50/0,5 ml)	0,5	0,5	0,5	0,5	0,5	0,5	0,5
	Incubation 18 h à + 4°C						
Globules rouges sensibilisés	4,0	4,0	4,0	4,0	4,0	4,0	4,0

(1) Pepsine cristallisée Merck (N° 7192).

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La solution est immédiatement soumise à une filtration stérilisante puis incubée à 37°C pendant 22 heures. Le *p*H est alors ramené à 6,5 avec NaOH N/10 et on obtient ainsi une solution à environ 6,8 % de protéines qui est diluée à 6,2 % par addition d'eau distillée contenant du glucose, du glycocolle et du merthiolate.

Après filtration stérilisante (filtres Millipore 0,45 μ) la solution finale (à 5 % de protéines) est répartie en unité de 50 ml.

II. - MESURE DE L'ACTIVITÉ ANTI-COMPLÉMENTAIRE

La méthode que nous proposons s'applique particulièrement au contrôle des préparations de γ-globulines à 5 %.

Une telle méthode doit être basée sur un dosage précis du complément de cobaye par hémolyse à 50 %. Nous avons précédemment publié une méthode répondant à cet impératif [6]. Les principaux réactifs utilisés y sont décrits.

Préparation du complément de cobaye

Chaque lot est constitué d'un mélange d'au moins 20 sérums de cobayes mâles (sang prélevé par ponction cardiaque). Le mélange de

TABLEAU I

4	5	6	7	8	9	10	Témoin C'	Témoin GR	Blanc C'
0,3	0,4	0,45	0,46	0,47	0,48	0,49	0,5	1,0	4,5
0,2	0,1	0,05	0,04	0,03	0,02	0,01	—	—	—
0,5	0,5	0,5	0,5	0,5	0,5	0,5	0,5	—	0,5
h à + 4°C		Incubation 18 h à + 4°C							
4,0	4,0	4,0	4,0	4,0	4,0	4,0	4,0	4,0	—

sérums est absorbé à deux reprises et à 0°C avec des hématies de mouton selon les indications données par MAYER [7], le rapport du volume de sérum à celui du culot globulaire étant de 1/30. Après filtration stérilisante, le complément est réparti dans des tubes stériles et conservé à — 80°C. Le titrage du complément doit être répété à chaque emploi. Le taux moyen (selon des titrages effectués sur 16 lots) est de 285 unités C' H 50.

Préparation des γ-globulines à tester

Cinq ml de la solution finale de γ-globulines à 5 % sont dialysés pendant 24 heures à + 4°C contre 1 litre de tampon Veronal contenant de l'azoture de sodium à 0,1 °/₀₀. Le tampon est renouvelé après 12 heures de dialyse. A la fin de la dialyse le taux de protéines de la préparation est déterminé par la méthode du biuret [2].

Mise en œuvre de la réaction

La répartition des réactifs est faite selon le *tableau I*. L'utilisation de pipettes de qualité « précision » est nécessaire. On répartit successivement : le tampon Veronal, la protéine dialysée, le complément dilué à 2 unités C' H 50/0,5 ml.

En ce qui concerne la répartition de la solution de γ-globulines dialysée, nous pensons que la méthode des double dilutions est à proscrire : elle donne des résultats imprécis et nous avons constaté pour certaines préparations une augmentation du pouvoir anti-complémentaire par dilution préalable accompagnée d'agitation.

L'incubation doit toujours être effectuée à basse température (+ 4°C) et de façon prolongée (18 heures), car il s'agit, dans le cas des γ-globulines d'une inactivation non spécifique du complément, plus lente que la fixation spécifique (par un complexe antigène-anticorps) et plus marquée à + 4°C qu'à + 37°C.

Après cette incubation, on ajoute à chaque tube (sauf le tube « Blanc C' ») 4 ml de la suspension de globules rouges standardisée et sensibilisée de façon optimale [6]. Après incubation à 37°C pendant 30 minutes puis centrifugation à 3 000 t/mn pendant 15 minutes, la lecture est faite à l'œil nu par rapport aux tubes témoins. Le témoin C' doit être totalement hémolysé. Par contre, dans le tube témoin GR, on ne doit observer aucune hémolyse ou seulement une trace d'hémolyse. Le tube « Blanc C' » permet d'apprécier la coloration donnée au surnageant par la dilution du complément ajoutée (généralement

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négligeable). En cas de difficultés, on peut effectuer une lecture au spectrophotomètre en mesurant la densité optique des surnageants à 530 mμ.

L'activité anti-complémentaire est donnée par la plus faible quantité de protéines requise pour inhiber totalement l'hémolyse, donc par le nombre de mg de protéines contenus dans le dernier tube où l'on a observé l'absence complète d'hémolyse.

Exemple : absence complète d'hémolyse dans les tubes 1, 2, 3. Le pouvoir anti-complémentaire est mesuré par le taux de protéines contenu dans le tube 3 : pour les globulines à 5 % (= 50 mg de protéines/ml), ce taux est donc de $0{,}3 \times 50 = 15$ mg. Dans ce cas il faut donc 15 mg de protéines pour inhiber complètement 2 unités C' H 50 de complément de cobaye.

L'activité anti-complémentaire varie en sens inverse du taux de protéines requis pour fixer le complément.

On pourra constater que dans cette méthode, le nombre de mg de protéines mis en jeu varie de 25 mg (tube 1) à 0,5 mg (tube 10) lorsqu'on étudie une solution de γ-globulines à 5 %.

On peut facilement adapter la méthode à l'étude d'autre solutions de protéines en faisant varier les concentrations selon les besoins.

III. - RÉSULTATS

1) Activité anti-complémentaire des globulines standard (non traitées) : tous les lots de γ-globulines standard testés ont manifesté une activité anticomplémentaire élevée : < 1 mg.

2) Evolution de l'activité anti-complémentaire pendant l'incubation a pH 4 en présence de pepsine :

Exemple : *Lot* 68 avant traitement 1 mg, après 5 h d'incubation 2,5 mg, après 22 h d'incubation > 25 mg.

Lot 71 avant traitement 0,5 mg, après 22 h d'incubation > 25 mg.

3) Activité anti-complémentaire des préparations fraîches de γ-globulines intraveineuses : toutes ces préparations manifestent une activité anti-complémentaire négligeable puisque le taux de protéines

requis pour fixer 2 unités C' H 50 de complément de cobaye est toujours > 25 mg.

4) Evolution de l'activité anti-complémentaire après traitement : l'activité anti-complémentaire augmente après quelques mois (les préparations étant conservées à + 4°C).

Lot 71 préparation fraîche > 25 mg, après 5 mois 20 mg, après 7 mois 15 mg, après 8 mois 5 mg.

Lot 75 préparation fraîche > 25 mg, après 3 mois 15 mg, après 5 mois 10 mg, après 6 mois 10 mg, après 7 mois 10 mg.

Lot 80 préparation fraîche > 25 mg, après 2 mois > 25 mg, après 3 mois > 25 mg, après 5 mois 10 mg.

Lot 84 préparation fraîche > 25 mg, après 2 mois > 25 mg après 5 mois > 25 mg.

Par décret du 11 septembre 1969, le taux limite de l'activité anti-complémentaire des γ-globulines injectables par voie intra-veineuse a été fixé à 10 mg. Cette limite nous paraît très valable.

Les résultats donnés ci-dessus montrent que les préparations de γ-globulines intra-veineuses telles que nous les préparons conservent une activité anti-complémentaire compatible avec leur utilisation pendant au-moins 6 mois.

La durée exacte de validité d'un lot de globulines intra-veineuses ne peut pas être fixée à l'avance. L'activité anti-complémentaire doit être contrôlée, à notre avis, tous les deux mois pour une préparation fraîche, puis tous les mois lorsque cette activité tend à augmenter (dès que le taux devient inférieur à 25 mg).

5) Tolérance clinique des γ-globulines intra-veineuses : une expérimentation complète est actuellement en cours. Les premiers résultats montrent que les préparations dont l'activité anti-complémentaire est faible (> 10 mg) sont bien tolérées à condition d'observer quelques précautions déjà soulignées par d'autres [3] : injection lente, surtout à la première perfusion.

RÉSUMÉ

Une technique de mesure de l'activité anti-complémentaire des préparations de γ-globulines intra-veineuses est décrite permettant de déterminer avec précision le nombre de mg de protéines inhibant totalement 2 unités C' H 50 de complément de cobaye. Pour une pré-

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paration fraîche, le taux doit être supérieur à 25 mg. L'activité anti-complémentaire augmente après quelques mois.

Quatre lots de γ-globulines intra-veineuses préparés selon BARANDUN et coll. [3] ont été suivis : les résultats montrent que l'activité anticomplémentaire compatible avec leur utilisation se maintient pendant 6 mois au moins. Le taux d'activité anticomplémentaire doit cependant être contrôlé régulièrement et ne doit pas devenir inférieur à 10 mg.

SUMMARY

A method for determination of the anticomplementary activity of the intravenous gamma globulins is outlined. It is possible to determine precisely the number of mg of proteins for the total inhibition of 2 U C' H 50 of guinea-pig complement. For a fresh preparation, there must be more than 25 mg. The anticomplementary activity increases after several months.

Four batches of intravenous γ globulins prepared according to Barandum and coll.'s method were studied ; the results showed that the anticomplementary activity compatible with their use is conserved for at least 6 months. However, the anticomplementary activity must be controlled regularly and must not be lower than 10 mg.

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Centre de Transfusion Sanguine,
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EXHIBIT 15

US005410025A

United States Patent [19]

Moller et al.

[11] Patent Number: 5,410,025

[45] Date of Patent: Apr. 25, 1995

[54] **UNMODIFIED INTRAVENOUSLY ADMINISTERED IMMUNOGLOBULIN PREPARATIONS CONTAINING IMMUNOGLOBULIN M AND/OR A**

[75] Inventors: Wolfgang Moller, Oberursel; Detlef Piechaczek, Munster, both of Germany

[73] Assignee: Biotest Pharma GmbH, Dreieich, Germany

[21] Appl. No.: 154,149

[22] Filed: Nov. 18, 1993

Related U.S. Application Data

[63] Continuation of Ser. No. 782,747, Oct. 18, 1991, abandoned, which is a continuation of Ser. No. 561,037, Aug. 1, 1990, abandoned.

[30] Foreign Application Priority Data

Aug. 17, 1989 [DE] Germany 39 27 111.0

[51] Int. Cl.[6] A61K 35/14; C07K 3/12

[52] U.S. Cl. 530/390.5; 530/387.1; 530/412; 530/416

[58] Field of Search 530/389.1, 368, 369, 530/387.1, 412, 416, 390.5

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Primary Examiner—David L. Lacey
Assistant Examiner—F. C. Eisenschenh
Attorney, Agent, or Firm—Sprung Horn Kramer & Woods

[57] ABSTRACT

Intravenously administered chemically unmodified immunoglobulin preparation containing more than 5% of its total immunoglobulin by weight consisting of IgM and/or more than 10% of its total immunoglobulin of IgA and with a low anticomplementary activity, and method of preparing it by anion-exchange chromatography.

8 Claims, No Drawings

UNMODIFIED INTRAVENOUSLY ADMINISTERED IMMUNOGLOBULIN PREPARATIONS CONTAINING IMMUNOGLOBULIN M AND/OR A

This application is a continuation, of application Ser. No. 782,747, filed Sep. 18, 1991, now abandoned which is a continuation of application Ser. No. 561,037, filed Aug. 1, 1990, now abandoned.

The present invention relates to intravenously compatible immunoglobulin preparations that have not been chemically modified and that contain more than 5% immunoglobulin M (IgM) and/or more than 10% immunoglobulin A (IgA) in terms of their total immunoglobulin content.

The immunoglobulins are a group of glycoproteins that occur in the body in response to the appearance of foreign antigens and are responsible for attacking and eliminating them. Although there are several classes of immunoglobulins, only IgG, IgA, and IgM occur in the plasma in significant concentrations.

At approximately 12 mg/ml, IgG is the major immunoglobulin in the plasma. It is mainly responsible for combating viral infections. The concentration of IgM is definitely lower—approximately 1.5 mg/ml. IgM is mainly involved in combating bacterial pathogens and in picking up bacterial toxins. IgA has a mean plasma concentration of 3.5 mg/ml, has the responsibility of neutralizing various viruses, those of poliomyelitis, measles, and influenza for example, and is encountered in its secretory, dimeric form in the seromucous secretions.

Immunoglobulin preparations have been employed for passive immunotherapy for some 40 years. Most of these substances are pure IgG preparations, with only low levels of IgA and IgM. Immunoglobulin preparations, furthermore, could be administered only intramuscularly prior to the 1960's, and their painful side effects prevented the use of large doses.

Several intravenously applicable IgG preparations were subsequently developed either by modifying the immunoglobulin chemically or enzymatically or by other methods.

IgG preparations, however, that also contained significant levels of IgM and/or IgA—those described in German Patent 2 404 265 or U.S. Pat. No. 3 808 189 for example—were still applicable only intramuscularly.

The first and up to now only intravenously applicable immunoglobulins to contain IgM and/or IgA are described in European Patent 0 013 901 and in German OS 3 825 429. Both of these preparations are essentially rendered intravenously applicable by being chemically modified with β-propiolactone (BPL). One measure of the intravenous compatibility of immunoglobulin preparation is the anticomplementary activity (ACA) described by E. Kabat and M. Mayer in Experimental Immunochemistry, 2nd ed., 1964, Springfield, Ill., Thomas Brooks, 133–240.

The object of the present invention is to develop immunoglobulin preparations that contain IgM and/or IgA, that have a low anticomplementary activity, and that are intravenously applicable although not chemically modified.

This object is attained by treating an immunoglobulin solution that contains immunoglobulin M and/or immunoglobulin A with an anion exchanger, gradient-eluting a fraction with a low anticomplementary activity, and optionally subjecting the fraction to a brief treatment at low pH and/or high temperature.

It has been surprisingly discovered that anion-exchange chromatography will attach the fraction responsible for the high anticomplementary activity so securely that subsequent elution under appropriate condition will wash out approximately 95% of the immunoglobulins collected, including most if the IgM and IgA with a low anticomplementary activity.

If the starting material has an elevated anticomplementary activity, the additional brief treatment of the eluate at low pH and/or high temperature will lower it to a level that is normal for intravenously applicable products.

Also surprising was the discovery that using a starting material with a low anticomplementary activity will sometimes make it possible to lower the anticomplementary activity just by treating the eluate from the anion exchanger at a low pH and/or high temperature with no need to remove some of the IgA and IgM through chromatography.

It will be obvious that the starting material for the anion-exchange chromatography should be produced under conditions that will protect the protein as much as possible against denaturation. Appropriate starting materials are solutions that contain immunoglobulin—Cohn Fraction II/III or Cohn Fraction III, other plasma fractions that contain IgA or IgM, such other solutions as milk or milk fractions, other body fluids, or residues from cultures of cells that produce IgA and/or IgM for example.

A fraction—Cohn Fraction II/III or III for example—that contains immunoglobulin can for example be dissolved in a buffer, and most of the impurities eliminated by precipitation with 0.5 to 5% octanoic acid at a pH of 4 to 6 and preferably 5. The solution is then treated at a low conductivity with an anion exchanger, attaching most of the IgA and IgM. Adsorption can be carried out batchwise, in a chromatography column, or on membranes.

If the desired product is to contain IgA and IgM the elution is carried out with a salt gradient that will leave approximately 10 to 20% of the IgM on the matrix. The precise eluting conditions depend on the type of anion exchanger and range from 10 to 400 m_{osm}, depending on the matrix and pH. The specifications for specific matrices are cited in the examples.

If the product is intended to contain IgA and not IgM, the elution is carried out at a lower osmolarity and the IgM will remain adsorbed onto the matrix. Depending on the chromatographic conditions, the eluate will contain 30 to 60% IgA and 70 to 40% IgG. The IgA can be further purified by appropriate measures.

If on the other hand the eluate also contains significant levels of IgM, the anticomplementary activity can be further decreased by 1 minute to 24 hours of additional treatment at a low pH, preferably 4 to 4.5, and/or at a higher temperature, 40° to 60° C. and preferably 50° to 54° C.

A pure IgM solution, extensively free of IgA, can be obtained by washing the anion exchanger ahead of time with a buffer to elute the IgA before the IgM.

When the anticomplementary activity in the starting material is lower, all of the IgM fraction that is adsorbed onto the anion exchanger can sometimes be eluted. In this situation, treating the eluate for 1 minute to 4 hours at a low pH, preferably 4 to 4.5, and/or at a high temperature, 40° to 60° C. and preferably 50° to 54°

C., will be sufficient by itself to reduce the activity to a tolerable level.

The solution can then be concentrated by ultrafiltration and its electrolyte level adjusted to that of the final intravenous formulation by diafiltration. The anticomplementary activity of the final product will then be in the range conventional for common commercial intravenous-IgG preparations or for the chemically modified IgM preparation Pentaglobin.

Since the anticomplementary activity of the IgG fraction not attached to the anion exchanger is also very low, the fraction can be employed in conjunction with the fractions that contain IgA and/or IgM in accordance with the invention to prepare mixtures that can be converted into intravenously compatible immunoglobulin preparations with a low anticomplementary activity and a desired ratio of IgG, IgA, and IgM. An immunoglobulin preparation that contains IgM and IgA and has the same composition as the commercial, chemically modified Pentaglobin—80% IgG, 10% IgA, and 10% IgM—for example but with an anticomplementary activity that is equal or lower can be prepared.

The immunoglobulin preparations that contain IgM and/or IgA in accordance with the invention can be subjected before or after the steps of the method in accordance with the invention to such in-themselves known sterilization procedures as treatment with β-propiolactone and ultraviolet light, treatment with solvents and/or detergents, or pasteurization.

The invention will now be described with reference to examples without being limited to them.

EXAMPLE 1

10 kg of Cohn paste III were dissolved in 50 l of 0.1M acetate buffer at a pH of 5 and treated with 1.5 kg of octanoic acid. The precipitate was centrifuged out after 4 hours and the supernatant dialyzed against 20 mM of piperazine and 20 mM of sodium chloride at a pH of 6. The solution is then applied onto a 5-l column of TMAE-Fraktogel (Merck, Darmstadt) equilibrated with the same buffer and chromatographed in 5 runs. The unattached IgG fraction was collected and concentrated by ultrafiltration.

An IgA-rich fraction was eluted with 20 mM of piperazine and 100 mM of sodium chloride at a pH of 6 and an IgM-rich fraction with 20 mM of piperazine and 150 mM of sodium chloride at a pH of 6. The rest of the attached protein was then washed out of the column with 20 mM of piperazine and 190 mM of sodium chloride at a pH of 6.

Table 1 shows the fraction's composition and anticomplementary activity.

TABLE 1

	IgG g/l	IgA g/l	IgM g/l	ACA CH 50/ml
IgG fraction	50	0.3	0	9
IgA fraction	31	18	0.1	17
IgM fraction	9	9	31	41
residual fraction	8	3	39	441

A chemically unmodified immunoglobulin preparation comprising 80% IgG, 10% IgA, and 10% IgM was then mixed from the IgG, IgA, and IgM fractions in this example and compared with the commercial product Pentaglobin, which is modified with β-propiolactone (Table 2).

TABLE 2

	IgG g/l	IgA g/l	IgM g/l	ACA CH 50/ml
Invention preparation:	40.0	4.9	5.0	13
Pentaglobin, Batch 1462019 (reference):	43.4	4.2	5.0	26

EXAMPLE 2

1 kg of Cohn Paste III was treated as described in Example 1. It was applied onto a chromatography column and immediately eluted with 20 mM of piperazine and 160 mM of sodium chloride at a pH of 6. The fraction contained 50% IgG, 23% IgA, and 27% IgM in terms of the overall immunoglobulin content. The anticomplementary activity was 26 CH 50/ml.

EXAMPLE 3

1 kg of Cohn Paste III was treated as described in Example 1 and passed in two runs through a 2 l column of QMA-Accell. It was immediately eluted with 20 mM of piperazine and 20 mM of sodium chloride at a pH of 4.7. The fraction contained 38% IgG, 27% IgA, and 35% IgM in terms of the overall immunoglobulin content. The anticomplementary activity was CH 50/ml and decreased to 20 CH 50/ml subsequent to 30 minutes of treatment at a pH of 4.0.

EXAMPLE 4

10 kg of Cohn Paste II/III were treated as described in Example 1. Since Paste II/III contains more IgG than Paste III does, the level of octanoic acid was decreased to 0.75 kg. Table 3 illustrates the properties of the eluates.

TABLE 3

	IgG g/l	IgA g/l	IgM g/l	ACA CH 50/ml
IgG fraction	49	1	0	4
IgA fraction	35	15	0.2	7
IgM fraction	9	10	3.0	19
Remaining fraction	10	4	36	193

EXAMPLE 5

1 kg of Cohn Paste III was treated as described in Example 1. It was applied onto the chromatography column, washed with 20 of piperazine and 120 mM of sodium chloride at a pH of 6, and eluted with 20 mM of piperazine and 175 mM of sodium chloride at a pH of 6. The eluate was concentrated by ultrafiltration and adjusted by diafiltration to a protein level of 50 g/l in 75 mM of sodium chloride and 2.5% glucose at a pH of 7. Table 4 illustrates the preparation's properties.

TABLE 4

	Protein g/l	IgM g/l	ACA CH 50/ml	Rec. Titer[2] *Ps. aerug.*
IgM preparation (invention)				
After chromatography	54.3	50.5	2300	40 960
After 20 min. @ 50° C.	54.3	50.5	29	40 960
After freeze-drying[1]	50.7	46.5	27	40 960
Pentaglobin				
Batch 1462019	53.3	5.0	26	1 280

TABLE 4-continued

	Protein g/l	IgM g/l	ACA CH 50/ml	Rec. Titer[2] *Ps. aerug.*
(reference)				

[1]Reconstituted with distilled water.
[2]The antibacterial titer against *Pseudomonas aeruginosa* was determined by passive hemagglutination as described by E. Neter, Bact. Rev. 20, 166 (1956).

EXAMPLE 6

6 kg of Cohn Paste III were treated as described in Example 1. It was applied onto a chromatography column, washed at each of 3 runs with 20 mM of piperazine and 120 mM of sodium chloride at a pH of 6, and eluted with 20 mM of piperazine and 175 mM of sodium chloride at a pH of 6. The three IgM eluates were combined, and 3 l of the resulting 12 were treated as described in Example 5.

The other 9 l were irradiated in a rotary-circulation apparatus with two 20 W ultraviolet lamps at 600 rpm and a throughput of 20 l per hour at a distance of 1 cm.

Subsequent to concentration to 40 g of protein/l by ultrafiltration, half of the solution was treated with 0.05% β-propiolactone for 90 minutes at a pH of 7.2 and a temperature of 25° C.

The other half of the solution was treated for 4 hours at 25° C. with 0.3% tri-N-butyl phosphate (TNBP) and 1% Tween 80. Both batches were then treated as described in Example 5.

The two preparations were adjusted to the same protein and IgM contents. Table 5 illustrates the anticomplementary activity and the antibacterial titer.

TABLE 5

	ACA CH 50/ml	Rec. Titer *Pseudomonas aeruginosa*
Unsterilized	32	20 480
Sterilized with UV & BPL	30	20 480
Sterilized with UV, TNBP, and Tween	31	20 480

Subject to the conditions acceptable for adequate inactivation of human-pathogenic viruses, neither the anticomplementary activity nor the antibacterial action of the IgM preparation altered significantly.

The immunoglobulin preparations in accordance with the invention, which can be made available as is or optionally in the form of solutions that must be diluted before injection or freeze-dried, can also contain additional proteins (human albumin for example), sugars (glucose for example), amino acids, or appropriate monoclonal antibodies.

It will be appreciated that the instant specification and claims are set forth by way of illustration and not limitation, and that various modifications and changes may be made without departing from the spirit and scope of the present invention.

We claim:

1. A process for preparing a polyclonal chemically unmodified immunoglobulin preparation wherein at least 5% by weight of all the immunoglobulin it contains is IgM, which is low in anticomplementary activity, and which is directly adminstrable intravenously, from polyclonal material which is either a chemically unmodified human Cohn fraction II/III or a chemically unmodified human Cohn fraction III that contains immunoglobulins which comprises passing the polyclonal material through an anion exchange column under conditions to absorb the immunoglobulins to the column, passing through said column an eluant, wherein said eluant comprises a sodium chloride gradient with a concentration of 20 mM to 175 mM and a buffer under acidic conditions, such that high anticomplementary activity immunoglobulins remain bound to the column while low anticomplemetary immunoglobulins are selectively eluted, and collecting the eluate containing said low anticomplementary immunoglobulins.

2. The method according to claim 1, wherein the initial polyclonal material or the eluate is heated to 40° to 60° C. for 1 minute to 24 hours.

3. The method according to claim 1, wherein the initial polyclonal material or the eluate is incubated at a pH of 3.5 to 5 for 1 minute to 24 hours.

4. The method according to claim 1, wherein the anion exchanger is a polymer with a TMAE (trimethyl amino ethyl) group or a QMA (quarternary amino ethyl) group.

5. The method according to claim 1, wherein the anion exchanger is a polymer with a DEAE (diethyl amino ethyl) group.

6. The method according to claim 1, wherein the buffer comprises 20 mM of piperazine.

7. The method according to claim 1, wherein the eluant has a pH in the range of 4.7 to 6.

8. The method according to claim 1, wherein the anion exchanger is a member selected from the group consisting of

a) a copolymer of the primary monomer N-Acryloyl-2-amino-2-hydroxy-methyl-1,3-propandiol and a trimethylaminoethyl-derivative of the monomer, and

b) a natural polysaccharide with quaternary amino groups.

* * * * *

EXHIBIT 16

Vox Sang. 29: 101–123 (1975)

Anticomplementary Activity of Human Immunoglobulin G

I. Mechanism of the Artifactual Increase in Anticomplementary Activity of IgG During the Assay

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Abstract. The anticomplementary activity of IgG can be increased up to 20-fold by pipetting during the preparation of serial dilutions for the assay of this activity. Albumin, if added to the IgG solution before the serial dilutions, completely prevents this artifactual increase in activity. Polyethylene glycol, polyvinyl pyrrolidone, methyl cellulose, gelatin and octanol are also effective stabilizers of IgG.

Repeated pipetting of IgG solutions caused marked linear increase in their anticomplementary activity. The formed anticomplementary activity was due to small amounts of highly aggregated protein. The amount of activity formed depended on at least four factors: [1] the number of pipetting steps; [2] the IgG concentration; [3] the level of albumin or other stabilizers, and [4] the pH, which influences the stabilization by albumin.

The anticomplementary activity of IgG is increased up to 100-fold by exposure to gas-liquid, liquid-liquid and hydrophobic solid-liquid interfaces. Albumin and polyethylene glycol prevent the activity increase during these treatments. The tendency of IgG to aggregate at interfaces and the ability of albumin and other substances to prevent the aggregation paralleled their rate of adsorption to the air-water interface. Solutions of dansyl chloride in decane emulsified in aqueous solutions of IgG and albumin specifically label the proteins at the liquid-liquid interfaces. The mechanism of stabilization can be explained by preferential adsorption of surface-active proteins and polymers.

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[2] The authors wish to thank Mr. T. ZUBER for technical assistance during a portion of this study, and Dr. F. SKVARIL for assistance in the preparation of the manuscript. Surface pressure measurements were carried out with equipement kindly provided by the Department of Pharmacology, University of Berne. The authors are indepted to Dr. PHILIP MORSE for helpful discussion and assistance in these studies.

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Introduction

Many investigators have documented that isolated IgG has marked anticomplementary properties [11, 12, 28]. The anticomplementary components in IgG preparations have been shown to be aggregates [10, 15, 16, 18, 19, 32] which form spontaneously or as a result of the isolation procedures, and which can also be generated by heating [15, 18, 19], chemical coupling [19] or exposure to organic solvents [33].

The presence of anticomplementary aggregates limits the clinical applications of immunoglobulin preparations. Intravenous administration of such preparations can cause serious systemic reactions [1]. Intramuscular injections, while being better tolerated, are in many indications significantly less effective. Various treatments (e. g., low pH, proteolysis, chemical modification) have been used to abolish the anticomplementary properties of IgG and render it safe for intravenous administration. However, the treated preparations generally have decreased stability or lessened biological effectiveness. These shortcomings have prompted the search for ways to prevent or control the aggregation of IgG during its isolation and storage.

DAVIS *et al.* [11] reported in 1944 that albumin and other serum fractions decrease or abolish the anticomplementary activity of IgG. They assumed that albumin somehow inhibits the inherent anticomplementary properties of IgG. The mechanism of this inhibition remained obscure. Recently, in what appeared to be an unrelated observation MALGRAS *et al.* [24] reported that serial dilution of IgG solutions sometimes caused increased anticomplementary activity and gave falsely high results when used in assays for this activity.

In routine measurements of anticomplementary activity in this laboratory, we observed unusual patterns of complement fixation with some IgG samples. IgG which had been treated at pH 4 was not anticomplementary at high concentrations but showed sharply increased anticomplementary activity when serially diluted more than 1 : 32. When monomeric IgG obtained by gel filtration was tested, the undiluted solution was not anticomplementary, but 1 : 2, 1 : 4 and 1 : 8 serial dilutions of it completely inhibited added complement. The present investigations were undertaken to find the reason for these anomalous complement binding patterns. The results indicated that pipetting of IgG serial dilutions during the assay causes a marked increase in the anticomplementary activity and that this increase is prevented by albumin and a variety of other substances. The results of an extensive

study of the effect of pipetting on IgG solutions are reported. Furthermore the effect of exposure of IgG to interfaces and the influence of surface active substances were studied.

Materials and Methods

Proteins. γ-globulin fraction II analogue was obtained from human plasma by the Cohn cold ethanol fractionation as modified by KISTLER and NITSCHMANN [21]. Lyophilized powder was dissolved in water as a 12- to 14-percent solution, sterile-filtered and stored at −20 °C or +4 °C. These preparations, further designated as fraction II, were found to consist of 96–98% γ-globulins, 2–3% albumin and traces of α- and β-globulins when analyzed by zone electrophoresis on cellulose acetate. Upon micro-immunoelectrophoresis [31] using horse antiserum to whole human serum, precipitin arcs corresponding to IgG, albumin, IgA, IgM and other α- and β-globulins were visible. Four lots of fraction II were studied.

Fraction II which had been treated at pH 4 with pepsin 1:10,000 by the method of BARANDUN *et al.* [1] was supplied by the Central Laboratory of the Swiss Red Cross, Berne. Gelatin (Physiogel) and albumin (Cohn Fraction V analogue) were also Swiss Red Cross products. Albumin was defatted with charcoal at low pH [9].

Polymers and chemicals. Polyethylene glycols (PEG) with molecular weights of 1,000, 4,000 and 20,000 were practical grade from Fluka, Buchs, Switzerland. Polyvinyl pyrrolidone (PVP), methyl cellulose, 25 cP (MC), dextran and sodium heparinate (pure grade) and 1-octanol (analytical grade) were also from Fluka. Decane, olefin-free, *purum*, was obtained from Fluka. Dansyl chloride (1-dimethylaminonaphtalene-4-sulfonyl chloride, DNS Cl) was purchased from Calbiochem, Luzern, Switzerland. All other chemicals were analytical grade reagents from Merck, Darmstadt, FRG.

Glass beads. Glass beads (Ballotini) with average diameter of 0.9 mm and surface area of 3.8×10^{-2} cm^2 were treated with sulfuric acid saturated with potassium dichromate, and were thoroughly rinsed with twice-distilled water and air-dried before use. Beads were siliconized by five treatments with Siliclad (Clay Adams, Parsipany, N. J.) according to the manufacturers directions.

Preparation of purified IgG. For some experiments fraction II was further purified by DEAE-cellulose chromatography (Whatman DE 52) in 0.01 M sodium phosphate buffer, pH 7.8 [13]. The protein which emerged in the drop-through volume, representing about 80% of the applied protein, was pooled. This protein formed a single arc in the γ-region when tested at 30–40 mg/ml by micro-immunoelectrophoresis using horse anti-human serum and rabbit anti-human IgG. No IgA, IgM or albumin were detectable. In the text, this preparation is designated as IgG. Similar chromatographic conditions have been shown to remove a portion of IgG having fast-gamma electrophoretic mobility [13].

Exclusion chromatography on Sephadex G-150. Protein samples were fractionated on a column of Sephadex G-150 (5.0×85 cm) equilibrated in 0.02 M sodium phosphate buffer, pH 6.6, containing 0.13 M sodium chloride. The column was operated from top to bottom. Flow rate was maintained at 40 ml/h and 10-ml fractions were collected.

Protein concentrations. Protein content was determined by Kjeldahl analysis or by measurement of absorbance at 280 nm using 13.5 as the value for $A\ \frac{1cm}{1\%}$ 280 nm for IgG.

Anticomplementary activity. Anticomplementary activities were assayed by the modified method of KABAT and MAYER [20] routinely used in the Swiss Red Cross. The diluent employed throughout was veronal-buffered saline (VBS), pH 7.3, containing optimum levels of Ca^{++} and Mg^{++} [20]. Sheep blood was collected in Alsever solution and stored for at least 1 week at +4°C. Fresh frozen guinea pig serum purchased from the Institute for General Microbiology, Bern, was used as the source of complement. Each lot was divided into 1-ml aliquots and stored at −30°C. Standardization of complement and sensitization of washed sheep erythrocytes were performed as recommended by KABAT and MAYER [20]. The complement levels in different lots of guinea pig serum ranged from 150 to 250 50-percent hemolytic units (CH50) per milliliter.

For titration of anticomplementary activity, two-fold serial dilutions (1 ml) of protein solution were made with VBS. 1 ml of diluted guinea pig serum containing 2 CH50 and 4.5 ml VBS were added to each dilution. Buffer blanks and complement controls containing 2 and 11 CH50 in a total volume of 6.5 ml were also prepared. The tubes were incubated at 37°C for 90 min. 1 ml of optimally sensitized erythrocyte suspension (5×10^8 cells/ml) was added and the samples were further incubated at 37°C for 60 min and cooled. The unlysed cells were removed by centrifugation and the optical densities of the supernatants were measured at 546 nm. Percent hemolysis was calculated relative to complement controls (2 CH50) taken as 100%. The anticomplementary titer was determined graphically as the exact dilution factor corresponding to 50% hemolysis. Specific anticomplementary activity, expressed as CH50/mg, is the reciprocal value of the weight of protein in this dilution.

Surface pressure. Surface pressure was measured by the WILHELMY [34] technique. A platinum plate with a perimeter of 39.6 mm, coated with platinum grey, was suspended from a transducing cell (Stratham, model UC2). A transducer/amplifier indicator (Hewlett-Packard, model 311A) amplified the signal which was recorded by a Philips 12-channel recorder. The teflon trough had dimensions of $10 \times 10 \times 1$ cm, and was equipped with sweeping bars for cleaning the gas-liquid surface. Buffers used for the subphase were 0.02 M sodium phosphate, pH 7.0, containing 0.15 M sodium chloride and 0.02 M sodium acetate, pH 3.98, containing 0.15 M sodium chloride. Proteins and polymers were equilibrated in the buffer. An aliquot (10–200 μl) of solution to be tested was injected into the subphase through a hole in the frame of the trough. The subphase was stirred magnetically. The initial rate of increase of surface pressure ($d\pi/dt$) was calculated from the maximum slope of the recorded curve. The equilibrium surface pressure ($\Delta\pi$) was estimated as the asymptote of the curve. Several subphase concentrations of each substance were tested in duplicate.

Dansylation at decane-water interface. The procedure for dansylation at liquid-liquid interfaces was derived from the method of BROWNE *et al.* [5]. Solutions of DNS Cl (2×10^{-3} M) in decane were prepared immediately before use and were filtered to remove any insoluble dansyl hydroxide. 2.5 ml of this solution and 5.0 ml of protein solution were stirred magnetically for 30 min. The phases were separated by centrifugation. Aliquots of the aqueous solution or suspension were spotted on paper or were subjected to electrophoresis on cellulose acetate strips. The strips were dried and viewed under ultraviolet light to detect fluorescence of the protein bands.

Results

In the assay of the anticomplementary activity as described in *Methods*, the first step is the preparation of serial dilutions of the protein solution to be tested. In some of our experiments we noticed that relatively minor variations in the technique of serial dilution caused large changes in the anticomplementary titers of fraction II solution. For each curve in figure 1, twofold serial dilutions were made by pipetting 1 ml of protein solution into a tube containing 1 ml of buffer, mixing the contents and then transferring 1 ml to the next tube. The technique of mixing and transfer to the next tube were different in each of the three experiments. The 50-percent hemolytic titers for the respective curves were 1:41, 1:690 and 1:990, which correspond to specific anticomplementary activities of 0.6, 9.9 and 14.1 CH50/mg. When a rubber bulb was used to draw the protein solutions into the pipette for mixing solutions, the results were the same as with normal mouth pipetting. In all of the following experiments the dilutions were made with a single pipette and mixed by pipetting, as in curve 3, figure 1.

When fraction II was assayed for anticomplementary activity by the described procedure (fig. 2, curve 1), the 50-percent hemolytic titer was 1:775, corresponding to an activity of 13.0 CH50/mg. When this fraction II solution was serially diluted, and then albumin was added to each dilution, the results remained unchanged (curves 2 and 3). When albumin was added before the solution was serially diluted, the titer was 1:19, or 0.32 CH50/mg (curve 4). With different albumin concentrations (8 and 22 mg/ml), the activity was consistently 0.3 ± 0.05 CH50/mg. Some other substances were found to possess a similar stabilizing property. In further experiments, PEG 4,000 was also used instead of albumin; other compounds will be characterized later on.

If the anticomplementary activity of fraction II was measured in the presence of albumin concentrations below a certain level, biphasic hemolysis curves were obtained, as shown in figure 3. The activities of fraction II without albumin (curve 1) and with 5 mg/ml (curve 5) were 6.8 and 0.2 CH50/mg, respectively. In curve 2 (0.5 mg/ml of albumin) the concentration of albumin was sufficient to prevent the formation of any anticomplementary activity up to the 1:8 dilution. With further dilution, the hemolysis decreased to 0% at 1:64, and then increased. A similar dip was seen in the hemolysis curves obtained with higher initial concentrations of albumin, 1 and 2 mg/ml (curves 3 and 4). With 5 mg of albumin, no dip occurred.

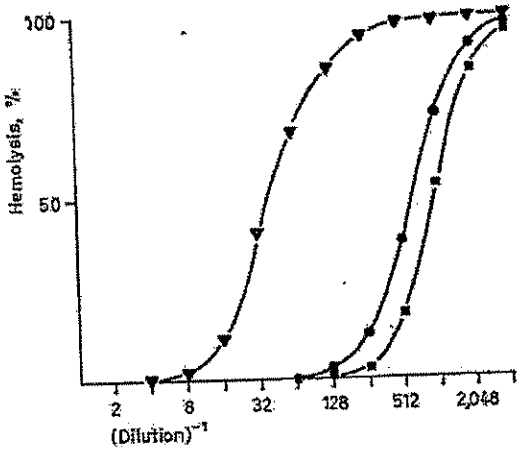

Fig. 1. Effect of different methods of serial dilution on the anticomplementary activity of fraction II. Two-fold serial dilutions (1 ml) of fraction II (70 mg/ml) were made with VBS diluent. Anticomplementary activities were measured as described in *Methods*. Curve 1 (▼): a separate 1-ml glass pipette was used for each serial transfer and the protein solution was mixed with VBS diluent by gently swirling the liquid in the tube; curve 2 (●): separate pipettes were used and each dilution was mixed by pipetting four times; curve 3 (■): a single pipette was used throughout and mixing was done by pipetting four times.

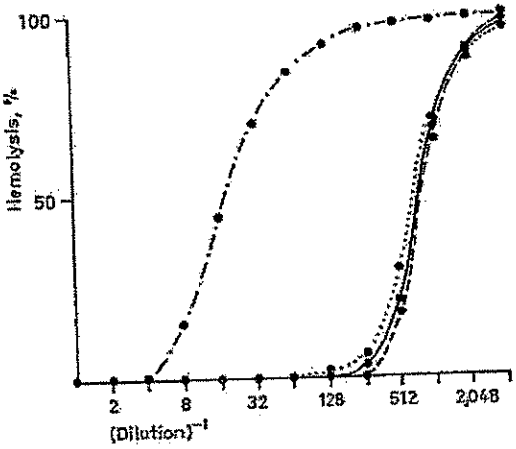

Fig. 2 Anticomplementary activity of fraction II without albumin and with albumin added before and after serial dilution. Curve 1 (——): fraction II (60 mg/ml) was serially diluted and assayed without albumin; curves 2 (...) and 3 (—·—): 1 ml of albumin solution, 2.2 mg/ml (curve 2) and 72 mg/ml (curve 3) were added to each dilution of fraction II; curve 4 (-·-·-): 1 ml of albumin solution (35 mg/ml) was added to 1 ml of fraction II solution (60 mg/ml) and the mixture was serially diluted and assayed.

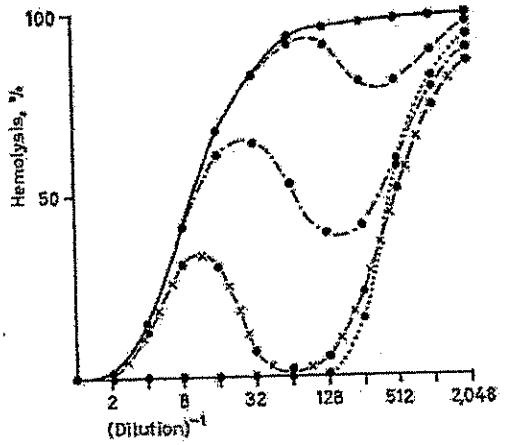

Fig. 3. Effect of different albumin concentrations on the anticomplementary activity of fraction II. Curve 1 (...): fraction II (35 mg/ml) was serially diluted and assayed without albumin; curves 2–5: Mixtures of fraction II (35 mg/ml) and albumin were serially diluted and assayed. Curve 2 (x-x-x); albumin, 0.5 mg/ml; curve 3 (- - -): 1 mg/ml; curve 4 (— —): 2 mg/ml, and curve 5 (——): 5 mg/ml.

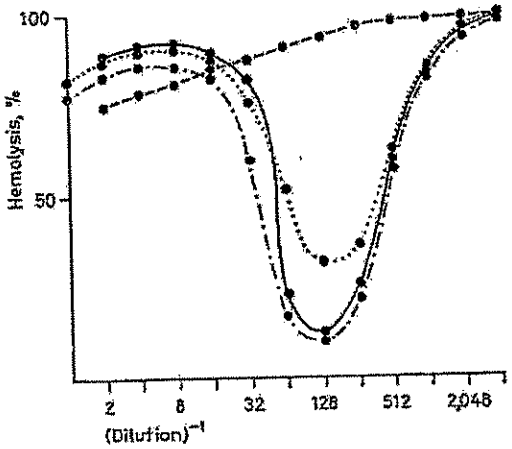

Fig. 4. Anticomplementary activity of pepsin (pH 4)-treated fraction II, assayed with and without albumin. Curve 1 (——): pH-4-treated fraction II (62 mg/ml) was serially diluted and assayed without albumin; curves 2(. . .) and 3 (- - -): 1 ml of albumin solution 70 mg/ml (curve 2) and 200 mg/ml (curve 3) was added to each dilution of pH-4-treated fraction II; curve 4 (— —): 1 ml of albumin solution (35 mg/ml) was mixed with 1 ml of pH-4-treated fraction II (62 mg/ml) before serial dilution.

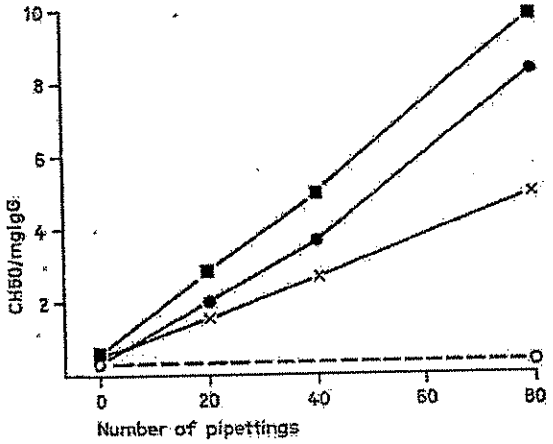

Fig. 5. Effect of repeated pipetting on the anticomplementary activity of fraction II. Aliquots of fraction II were pipetted with a 1-ml glass pipette 0, 20, 40 and 80 times. 1 ml of albumin solution (10 mg/ml) was then added to each sample and the anticomplementary activities were measured. Curves 1, 2 and 3 show the increase in anticomplementary activity with fraction II solutions of different concentrations. Curve 1 (■): IgG 2.2 mg/ml; curve 2 (●): 4.4 mg/ml; curve 3 (×): 17.5 mg/ml. Curve 4 (⊙) shows the activity of the same fraction II sample, 4.4 mg/ml; pipetted 80 times after the addition of albumin.

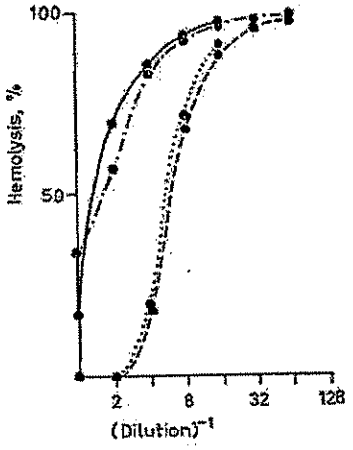

Fig. 6. Site of the anticomplementary activity formed by pipetting. 2-ml aliquots of fraction II (5 mg/ml) were tested as follows: Curve 1 (——): unpipetted; curve 2 (– –): pipetted 10 times with a single pipette; curve 3 (- - -): pipetted 10 times, each time with a separate pipette. PEG was added to the sample in curves 1–3 before serial dilution. Curve 4 (. . . .): the solution remaining in the 10 pipettes from curve 3 was collected by rinsing with 2 ml of a solution of PEG in saline and assayed.

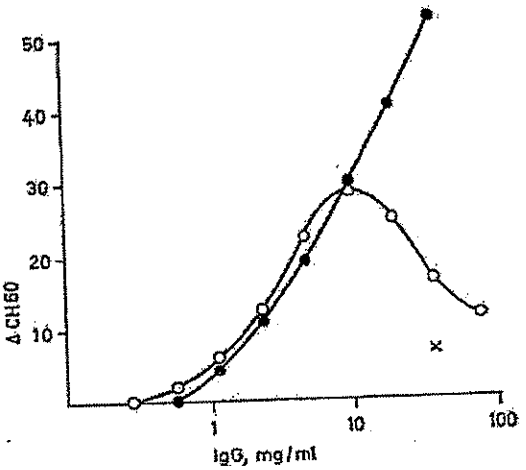

Fig. 7. Concentration dependence of the increase in anticomplementary activity with pipetting. Protein solutions were diluted to the designated concentrations. A 2-ml aliquot of each sample was pipetted 80 times. PEG was added and the activities were assayed. The increase in total anticomplementary activity was calculated as the reciprocal of the 50-percent hemolytic titer of the pipetted sample minus that of the corresponding unpipetted control. Curve 1 (○): fraction II; curve 2 (●): IgG. (A chromatographically purified sample of the same fraction II lot.) The activity of a mixture of IgG (37 mg/ml) and albumin (1 mg/ml) after 80 pipetting is designated by (×).

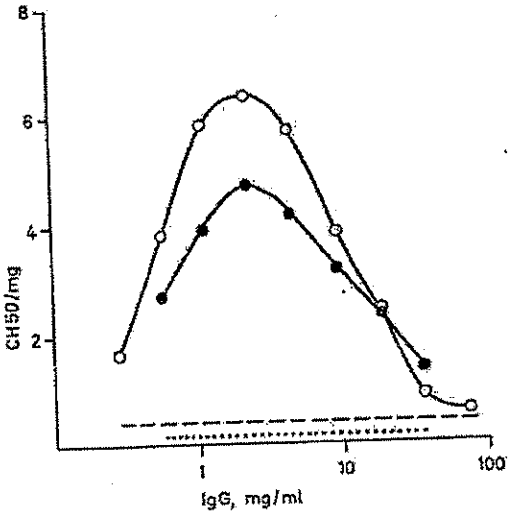

Fig. 8. Concentration dependence of the increase in specific anticomplementary activity (CH50/mg) with pipetting. The results in figure 7 are expressed as CH50/mg IgG. Curve 1 (○): fraction II; curve 2 (●): IgG. The dashed and dotted lines represent the activity (CH-50/mg) of unpipetted controls of Fraction II (—) and IgG (. . .).

When fraction II which had been treated at pH 4 with pepsin (1 :10'000) was assayed, a similar biphasic hemolysis curve was obtained (fig. 4, curve 1). Curves 2 and 3 show that this effect was not reversed if albumin was added after serial dilution of the sample. When albumin was added to this preparation prior to serial dilution (curve 4), no dip in the hemolysis curve was observed at any dilution. Ten different pH 4–pepsin-treated fraction II preparations gave similar results. This effect has also been observed with fraction II preparations which had been treated with plasmin, β-propiolactone or at pH 4 without pepsin to reduce their anticomplementary activity.

Figure 5 shows the effect of repeated pipetting with a single glass pipette on the anticomplementary activity of fraction II solutions of different concentrations. 2-ml aliquots were pipetted 20, 40 or 80 times with a 1-ml pipette. Then albumin was added to each sample to prevent any further activity increase during the preparation of serial dilutions and the activity was assayed. As an unpipetted control, 2 ml of each solution was mixed with albumin and assayed for anticomplementary activity. Samples of fraction II solution which were mixed with albumin or with PEG and then pipetted 80 times had the same activity as the unpipetted controls. In the samples repeatedly pipetted before the addition of albumin, the anticomplementary activity increased in a nearly linear fashion. A linear increase resulted with each fraction II concentration, but the level of attained activity differed.

The results of the next experiment (fig. 6) show that the site of activity increase is in the film of protein solution remaining in the drained pipette. When fraction II solution was pipetted 10 times, each time with a different pipette, the anticomplementary activity of the solution was not increased over that of an unpipetted solution. The solution remaining in the 10 pipettes was collected in 2 ml of saline containing PEG, and was found to have the same activity as a solution which was pipetted 10 times with a single pipette.

In the following experiment, the increase in the anticomplementary activity of fraction II and of IgG, formed by pipetting solutions of different protein concentrations was studied (fig. 7). At concentrations up to 10 mg/ml, the anticomplementary activity of both samples was increased to approximately the same extent. At higher concentrations, the increase in the activity of fraction II was significantly less than that of the IgG. IgG mixed with a small amount of albumin (IgG 37 mg/ml, albumin 1 mg/ml) was only slightly increased in activity when pipetted 80 times.

In figure 8, the data of figure 7 are expressed as increases in specific anticomplementary activity (CH50/mg). According to the results when solutions

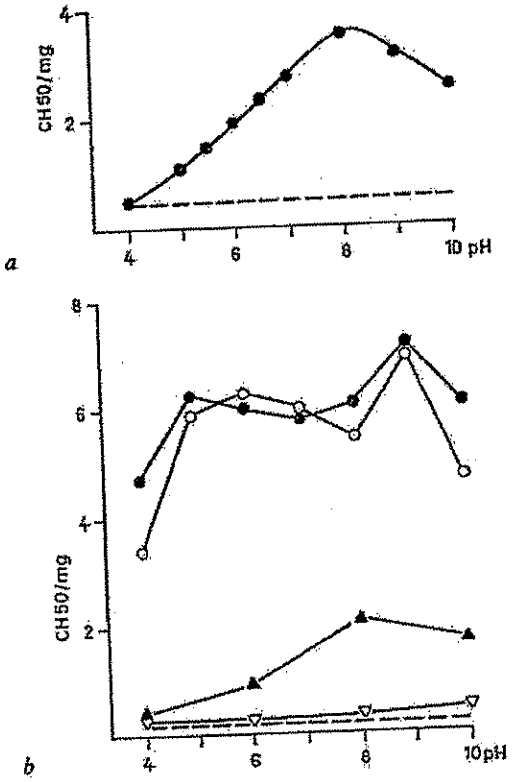

Fig. 9. pH dependence of the anticomplementary activity increase. *a* Solution of fraction II (18 mg/ml) were adjusted to the designated pH values. Two ml of each solution was pipetted 80 times, PEG was added and the solutions were neutralized and assayed (●). All samples were adjusted to the same concentrations before pipetting and after neutralization. Activity of unpipetted solution (---). *b* Samples of IgG, with and without added albumin, were pipetted 80 times at the designated pH values. The samples were then stabilized, neutralized and assayed as in fig. 9a. Curves 1 (●) and 2 (○): two different IgG samples, 10 mg/ml; curve 3 (▲): a mixture of IgG (10 mg/ml) and albumin (0.1 mg/ml); curve 4 (▽): a mixture of IgG (10 mg/ml) and albumin (0.3 mg/ml). The same preparation of IgG was used for curves 1, 3 and 4. Activity of unpipetted IgG (---).

of fraction II or IgG of different concentrations are pipetted 80 times, the largest increase in specific activity occurs in solutions containing about 2 mg/ml of protein.

The dependence of the increase in anticomplementary activity of pipetted fraction II on pH is shown in figure 9a. It can be seen that at pH 4 the protein solution did not change its anticomplementary properties, and that maximum increase in activity occurred in the pH region near 8. When two different

IgG preparations were tested in the same way, the pH dependence was not pronounced as with fraction II: only a slight difference between acidic and alcaline region could be observed (fig. 9b). The IgG sample containing 0.1 mg/ml albumin was shown to be pH-dependent similarly to fraction II. The activity of the same IgG sample containing 0.3 mg/ml albumin was not changed significantly at any pH value.

The data in table I show that the anticomplementary activity caused by pipetting is in the form of aggregates, most of which are too large to pass through a 0.2-μm membrane. If the solutions were membrane-filtered immediately after pipetting, 90–100% of the increase in anticomplementary activity formed by pipetting was removed. Filtration 15–20 min after pipetting removed less of the activity, indicating that the aggregates were dissociated on standing. The anticomplementary activity of unpipetted control solutions was not decreased by filtration. A slight decrease in protein concentration occurred in all samples. All of the preparations had previously been passed through 0.2-μm filters as a sterilization step.

Elution patterns of unpipetted and pipetted solutions of fraction II obtained by exclusion chromatography on Sephadex G-150, showed that repeated pipetting did not significantly alter the elution characteristics of the protein solution (fig. 10). Shortly after elution, aliquots from the fractions containing the highest protein concentration in each of the three peaks were stabilized with PEG and assayed for anticomplementary activity. The results are shown in table II, column A. The data in table II, column B, show the anticomplementary activity of the same fractions pipetted 80 times, and then stabilized with PEG and assayed.

Various substances were found to have a similar action on the anticomplementary activity of IgG to that of albumin. Low titers were obtained in the presence of gelatine, PEG with molecular weights of 1,000, 4,000 and 20,000, PVP, MC and 1-octanol (serial dilutions were made with octanol-saturated VBS diluent). As observed with albumin, low levels of these substances resulted in biphasic hemolysis curves. In the presence of dextran, heparin, methyl cellosolve, ethylene glycol, ethanol or glycine, IgG exhibited the same high anticomplementary titers as when tested alone. None of the substances tested enhanced or inhibited hemolysis by complement controls or caused hemolysis of erythrocytes in the absence of complement. Freshly thawed solutions of different preparations of fraction II had similar anticomplementary activity when measured in the presence of different stabilizers. This activity tended to increase during storage at 4 °C. Removal of impurities from fraction II by DEAE-cellulose chromatography generally resulted in a

Table I. Effect of membrane filtration on anticomplementary activity and protein content of unpipetted and pipetted IgG solutions

Protein solution tested	No. of pipettings per ml	Anticomplementary activity CH50/mg		Protein concentration mg/ml[1]	
		before filtration	after[2] filtration	before filtration	after[2] filtration
IgG	0	0.1	ND[3]	19.8	ND[3]
	40	10.8	1.0 (0 min)	19.9	18.2 (0 min)
			2.1 (15 min)		17.6 (15 min)
Fraction II	0	0.4	0.4	4.7	4.4
	40	4.8	0.4	4.7	4.4
Fraction II	0	0.7	0.7	4.7	4.5
	40	7.3	1.0 (0 min)	4.7	4.3 (0 min)
			1.4 (30 min)		4.4 (30 min)

[1] Protein concentrations were calculated from the mean value of two measurements of absorbency at 280 nm.
[2] Samples were membrane filtered immediately after pipetting, or 15 or 30 min later, where indicated.
[3] Not determined.

Table II. Anticomplementary activities of components I, II and III obtained by gel filtration of unpipetted and pipetted solutions of fraction II

Sample applied to Sephadex G-150	Component	CH50/mg	
		A	B
Unpipetted Fraction II Solution	I	3.0	18.5
	II	0.2	10.2
	III	0.2	6.2
Pipetted Fraction II Solution	I	10.8	40.2
	II	0.2	14.2
	III	0.2	9.0

Fractions representing the peak level of each component eluted from Sephadex G-150 (fig. 7) were tested shortly after elution. A 2.0-ml aliquot of the indicated fractions was mixed with PEG and assayed for anticomplementary activity (column A). Another aliquot was pipetted 80 times, mixed with PEG and then assayed (column B).

Table III. Increase in anticomplementary activity of IgG with various treatments

Treatment	Anticomplementary activity, CH50/mg		
	IgG	IgG+albumin	IgG+PEG
None	0.3	0.3	0.3
Pipetting 80 times	10.1	0.3	0.3
Bubbling with N_2, 40 min[1]	9.7	0.5	1.9
Stirring with decane, 40 min	36.5	4.3	8.1
Stirring with siliconized glass beads, 40 min	2.5	0.5	2.5
Stirring with glass beads, 40 min	0.6	0.9	0.5
Lyophilization	23.0	0.3	25.0

The IgG concentration in all solutions except those which were lyophilized was 5 mg/ml. In the second and third columns, albumin (10 mg/ml) or PEG (5 mg/ml) was added prior to treatment. In the solutions that were lyophilized, the concentration of IgG was 20 mg/ml, albumin 10 mg/ml and PEG 10 mg/ml.

[1] In 2 ml of protein solution N_2 was introduced through a teflon tubing (0.8 mm in diameter) with a speed of approximately 150–200 bubbles/min.

decrease in activity. This decrease may have been due to precipitation and removal of aggregates in the low ionic strength buffers used for dialysis and elution [19].

The striking increase in the anticomplementary activity of IgG occurs not only upon pipetting, but after various physical treatments as well (table III). The common feature of the first three treatments (pipetting, bubbling with nitrogen and stirring with decane) is the formation of interfaces of the air-liquid, liquid-liquid and solid-liquid type, and the exposure of the protein to these interfaces. During these treatments, the anticomplementary activity increased up to 100-fold. The treatments caused increased turbidity, and in some cases measurable loss of protein in the solution due to the precipitation. To compare the effects of hydrophobic and hydrophilic solid surfaces, siliconized and untreated glass beads (15 g, 180 cm^2 total surface area) were added to IgG solutions (6 ml, 5mg/ml) and slowly stirred for 40 min. The results (table III) indicate that more anticomplementary activity appeared

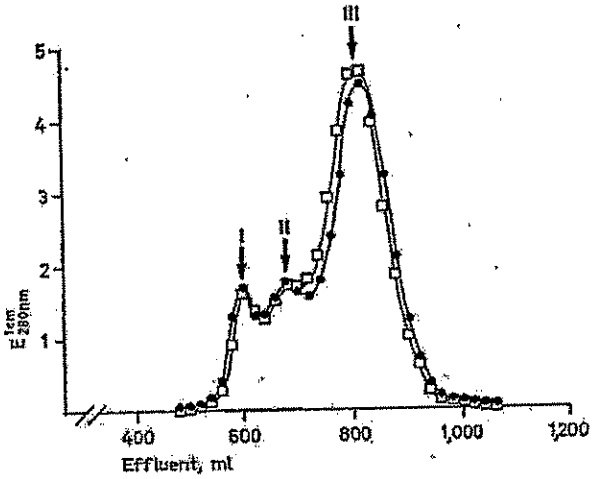

Fig. 10. Results of gel filtration of unpipetted and pipetted solutions of fraction II. Unpipetted (□) and repeatedly pipetted (●) solutions of fraction II (17 ml, 30 mg/ml) were applied to a 5 × 85 cm column of Sephadex G-150 equilibrated in 0.02 M sodium phosphate buffer, pH 6.6, containing 0.13 M sodium chloride. Flow rates were maintained at 40 ml/h and 10 ml samples were collected. The fractions designated I, II and III were taken for anticomplementary activity measurements shown in table III.

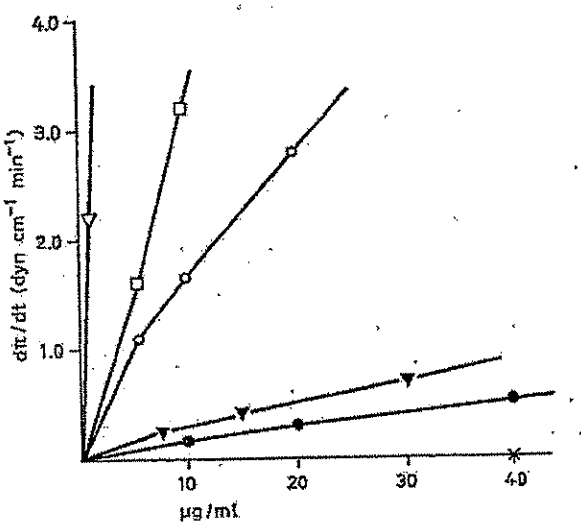

Fig. 11. Initial rate of increase in surface pressure caused by proteins and polymers. Aliquots of polymer solutions were injected into pH 7 subphase buffer in a Langmuir trough. The initial rate of surface pressure increase ($d\pi/dt$) was measured as described in *Methods*. ▽ = PEG; □ = PVP; ○ = albumin; ▼ = gelatin; ● = IgG; × = dextran, heparin.

in the solution upon stirring with the hydrophobic than with the hydrophilic beads. In columns 2 and 3 the effects of these treatments on IgG solutions containing albumin or PEG are shown. Less anticomplementary activity was formed during these treatments. Albumin (column 2) was more effective than PEG (column 3) in preventing the increase in anticomplementary activity caused by the hydrophobic surfaces of nitrogen, decane and siliconized glass beads. However, albumin did not prevent the small increase in activity caused by hydrophilic glass beads. During lyophilization there was no protection by PEG, but a complete protection by albumin. In some treatments the protective effect of PVP was also tested. This polymer (5 mg/ml) was completely effective in preventing the change with pipetting, was equivalent to albumin in protecting IgG upon stirring with decane, and was less effective than albumin or PEG during the nitrogen treatment.

Previous experiments showed that IgG is stabilized during various physical treatments not only by albumin and PEG, but by some other polymeric substances as PVP and gelatin; dextran and heparin were not effective. The data in figure 11 show the rates of increase in surface pressure at the air-liquid interface after solutions of these polymers were injected into a buffer subphase in a Langmuir trough. The rate of surface pressure increase ($d\pi/dt$, dyn cm^{-1} min^{-1}) can be considered an indication of the rate of accumulation of the substance at the interface. PEG, PVP, albumin and gelatin caused the surface pressure to increase more rapidly than did IgG over a range of concentrations (1–40 μg/ml). The equilibrium surface pressures for these substances at 10 μg/ml were: PEG, 5.9 dyn/cm; PVP, 3.5 dyn/cm; albumin, 12.5 dyn/cm; IgG, 5.5 dyn/cm. The equilibrium surface pressure of gelatin was not determined. Dextran and heparin did not cause any measurable change in the surface pressure.

In one of the previous experiments, the increase in anticomplementary activity of IgG with pipetting was shown to be less dependent of pH, than when albumin was present in the solution (fig. 9). At pH 4, albumin was more effective in preventing the increase in the activity. When various concentrations of IgG were injected into a pH 4 subphase buffer in the Langmuir trough, the rate of surface pressure increase was found to be nearly equal to that measured at pH 7. The rate of pressure increase caused by albumin at pH 4 was approximately 6 times faster than at pH 7. The equilibrium surface pressures of albumin and IgG were not changed at pH 4.

CECIL and LEWIS [8] reported that insulin was readily adsorbed at the surface of decane emulsion droplets. By incorporation of the hydrophobic protein reagent DNS Cl into the decane phase, BROWNE *et al.* [5] succeeded

Table IV. Fluorescence of proteins dansylated at decane-water interface

Composition of aqueous phase mg/ml		Fluorescence of aqueous phase after emulsification with solution of dansyl chloride in decane				
		spot test		acetate electrophoresis		
IgG	albumin	soluble	precipitate	albumin	IgG	precipitate
10	0	–	+++	–	–	+++
10	10	+++	–	+++	–	–
10	2	+++	–	+++	–	–
10	0.5	+++	–	++	–	–
10	0.1	++	±	±	–	±
0	0	–	–	–	–	–
0	10	+++	–	+++	–	–

For each experiment, 5 ml of protein solution or buffer was emulsified for 30 min with 2.5 ml of the hydrocarbon phase consisting of a solution of 2×10^{-3} M DNS Cl in decane. The phases were separated by centrifugation, and aliquots of the aqueous phase solution or suspension were tested by spoting on paper or by electrophoresis in cellulose acetate strips. Fluorescence of precipitated or soluble proteins was detected under UV light. +++=Strong fluorescence or precipitation; ++=moderate fluorescence or precipitation; +=slight fluorescence or precipitation; ±=very slight fluorescence or precipitation; –=no detectable fluorescence or precipitation.

in attaching a fluorescent label to the portion of the insulin molecule in contact with the hydrophobic phase. DNS Cl is poorly soluble in water and remains partitioned in the decane phase, where it is able to react only with residues in the interfacial region. This approach can also be used with aqueous solutions of more than one protein to determine if preferential adsorption of one protein occurs. The data in table IV show the results of preliminary experiments in which a solution of DNS Cl was emulsified in aqueous solutions of IgG and albumin. When IgG solutions were vigorously stirred with hydrophobic marker phase for 30 min, a portion of the protein became insoluble and highly fluorescent. The soluble protein did not show a visible fluorescence. When solutions containing IgG (10 mg/ml) and albumin (0.5, 1.0 and 10 mg/ml) were emulsified with DNS Cl solution, the aqueous phase became strongly fluorescent and no precipitate formed. After separation of the proteins by electrophoresis, only the albumin band

was highly fluorescent. With 0.1 mg/ml albumin and 10 mg/ml IgG, in addition to the labeled albumin some fluorescent precipitate formed. PEG also prevented the formation of fluorescent IgG precipitates, but did not prevent albumin from being labeled.

Discussion

MALGRAS *et al.* [24] previously reported that serial dilution could increase the anticomplementary activity of IgG solutions. In the present investigation we also found that different techniques of preparing serial dilutions of the same fraction II solution resulted in a wide range in the measured values for anticomplementary activity. Because the three assays in figure 1 were carried out identically except for the way in which the dilutions were mixed, the observed difference can only be explained as an increase in activity caused by pipetting. The data in figure 5 confirm these findings. Repeated pipetting before the assay is shown to cause a nearly linear increase in the anticomplementary activity of fraction II solutions. The same results were obtained with rubber bulb and with normal mouth pipetting, indicating that the increased anticomplementary activity is not due to contamination with antigens in the breath and formation of immune complexes with antibodies in the fraction II preparation. Pipetting *per se* must cause some changes of IgG molecules in solution to render them anticomplementary. Increased turbidity of the pipetted protein solutions first suggested that it might be aggregation. The results obtained when pipetted IgG solutions were membrane filtered were in agreement with this observation (table II). Because the anticomplementary activity formed by pipetting was almost completely removed and no significant decrease in the protein concentration could be detected, it is probable that small amounts of very large aggregates are responsible for the increased activity. Gel filtration experiments are in agreement with these results. Although no increase in the extent of aggregation could be seen in elution pattern of the pipetted sample (fig. 10), the activity of the excluded component which was respinsable for most of the anticomplementary activity, was increased (table II).

In some of the experiments, it was shown that albumin can prevent the increase in anticomplementary activity of fraction II caused by pipetting during (fig. 2) or before (fig. 5) the assay for this activity. Some investigators have already reported similar observations but the phenomenon remained unclarified. DAVIS *et al.* [11] found highanticomplementary titers for IgG

which had been isolated from serum by electrophoresis or by precipitation with sodium sulfate. When albumin or α- and β-globulins were added, the titers decreased eightfold or more. They suggested that these serum proteins might act by interfering with or reversing the interaction of IgG and complement. BECH [2] also observed that IgG became much less anticomplementary when incubated with albumin, human serum or animal serum. The results of DAVIS *et al.* [11] and of BECH [2] were both obtained with assayas in which the IgG solution was serially diluted. The low titers they observed in the presence of albumin or other serum proteins are consistent with the present observations and probably represent stabilization of IgG. The results in figure 2 (curves 2 and 3) show that albumin does not interfere with complement fixation.

Biphasic hemolysis curves were obtained when anticomplementary activity of fraction II was assayed in the presence of albumin concentrations below 5 mg/ml (fig. 3). Each curve appears to dip at the dilution where albumin level reached a minimum concentration necessary for complete stabilization of IgG under the experimental conditions (concentration of IgG, number of pipettings, number of serial dilutions). This level is in the range of 0.03–0.25 mg/ml for defatted albumin. Dips in hemolysis curves observed with pepsin (pH 4)-treated fraction II (fig. 4) indicate the presence of stabilizing factor(s) in these preparations. Some preliminary experiments indicated that these are the traces of IgG pepsin fragments and albumin.

In addition to albumin a variety of other substances was found to protect IgG against the alteration caused by pipetting. The values for the anticomplementary activity of a given IgG preparation measured in the presence of proteins (albumin, gelatin) or several synthetic polymers (PEG, PVP, MC) were in close agreement. Polysaccharides (dextran, heparin) did not protect IgG, but a partially methylated polysaccharide (MC) was a very effective stabilizer. Glycine, commonly used as a stabilizer of IgG solutions [15, 17, 23], did not protect IgG during serial dilutions. Of the low-molecular-weight substances tested, only 1-octanol was effective.

Buffers containing albumin or gelatin (0.1%) have been used in complement fixation test to prevent nonspecific hemolysis of erythrocytes [20] and adsorption of complement [7, 14] or protein antigens [26] to glass. The present results indicate that albumin or gelatin can also be used to prevent increase in anticomplementary activity when IgG solutions are pipetted. In preliminary experiments where 0.1% albumin or gelatin were added to the dilution buffer anticomplementary titers were two to six times higher than when albumin or gelatin were added to the initial IgG solution. The activity

may have been increased in the first serial transfer of IgG into the buffered albumin or gelatin solution. No stabilizer was present at this step.

Various physical treatments were found to cause the alteration of IgG molecules in solution. The anticomplementary activity was shown to be markedly elevated by exposure to surfaces of the gas-liquid, liquid-liquid and solid-liquid type (table III). It is well established that IgG is readily adsorbed to various types of surfaces [4]. When adsorbed to polystyrene [3, 27] and to bentonite [25], IgG increased anticomplementary activity. The adsorption of IgG to polystyrene is prevented if the surface is first exposed to albumin [3], gelatin [29], serum [3] or detergents [29].

The results obtained when fraction II solution was repeatedly pipetted each time with a separate pipette (fig. 6), suggest that the anticomplementary activity increase caused by pipetting is also due to exposure to interfaces. The solution adhering to the inner wall of the pipette is exposed to relatively large gas-liquid and glass-liquid interfaces. Because the anticomplementary material appeared in the liquid remaining in the pipette and not in the pipetted fraction II solution, it seems probable that the gas-liquid interface is the site of the activity increase with pipetting. With each successive pipetting, protein adsorbed at this interface is returned to the solution and a new gas-liquid interface is formed. The formation of the similar amount of anticomplementary activity with each pipetting is consistent with the linear increase in activity shown in figure 5.

KOCHWA *et al.* [22] have demonstrated that IgG is unfolded upon adsorption to polystyrene latex and that the extent of unfolding depends on the concentration of IgG at the surface. We found that the alteration of IgG caused by pipetting also depended on the concentration of the protein in solution. The increase in specific anticomplementary activity showed a sharp maximum near 2 mg/ml (fig. 8). This may represent the concentration at which the highest proportion of molecules are aggregated. Alternatively, the conformation or special arrangement of IgG molecules in aggregated form may be optimal for complement fixation. The difference in the total activity increase between fraction II and IgG (fig. 7) is probably due to the contaminant proteins which stabilize fraction II in concentrated solutions.

A good correlation was found between the ability of a given substance to protect IgG and the rate at which it accumulated at the air-liquid interface. Polymers with stabilizing ability (PEG, PVP, albumin and gelatin) accumulated at the air-liquid interface more rapidly than IgG and those which did not protect IgG (dextran and heparin) were not measureably adsorbed (fig. 11). The protection during other treatments in which IgG is

in prolonged contact with surfaces may depend on other characteristics such as rate of desorption, compactness of packing at the interface, equilibrium surface pressure, etc. PEG and PVP were more rapidly adsorbed than albumin but were less effective than albumin in preventing the aggregation of IgG with most treatments (table III). The equilibrium surface pressure of PEG and PVP were significantly lower than that of albumin.

No significant difference was found in the rate of adsorption of IgG at the air-liquid interface at pH 4 and at pH 7. This is consistent with the finding that IgG did not show a pronounced difference in the anticomplementary activity increase, when pipetted at different pH values. VAN OSS and SINGER [29] previously reported that the adsorption of IgG to polystyrene surfaces is independent of pH. This suggests that hydrophobic rather than ionic interactions are involved in adsorption at interfaces and in aggregate formation with pipetting.

A marked pH dependence was observed in the aggregation of IgG in the presence of albumin (fig. 9). The rate of adsorption of albumin at the air-liquid interface was found to be six times faster at pH 4 than at pH 7. BULL [6] showed that the adsorption of albumin to glass was maximum at pH 4–5. The increased surface activity of albumin is a possible explanation for its greater stabilizing activity at low pH values.

In a further attempt to clarify the mechanism of the protection of IgG by albumin, we used solutions of DNS Cl in decane to attach the fluorescent label to the interfacially adsorbed protein. Preliminary experiments showed that IgG was precipitated and labeled when solutions were emulsified with DNS Cl/decane solutions (table IV). The presence of albumin or PEG prevented both the precipitation and labeling of IgG at the interface. Neither IgG nor PEG prevented the labeling of albumin. These results suggest that albumin was selectively adsorbed from the mixtures and interfered with the adsorption, labeling and aggregation of IgG.

BERNHARD *et al.* [3] adsorbed an IgG solution repeatedly with polystyrene latex and found that less protein was adsorbed with each successive treatment. They interpreted this to mean that IgG was heterogeneous in its adsorption to the hydrophobic surface. PARKER and OSTERLAND [30] showed that human IgG myelomas differed in their ability to interact with hydrophobic probes. The hydrophobic binding sites were located in the Fab region and were probably at or near the exterior of the molecule. An extensive study of a series of well-defined myeloma proteins which is in progress, may show if surface behavior and aggregation are related to structural characteristics of a specific region of the IgG molecule.

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EXHIBIT 17

STORAGE

For the liquid preparation, store in a colourless glass container, protected from light. For the freeze-dried preparation, store in an airtight colourless glass container, protected from light.

LABELLING

The label states:

- for liquid preparations, the volume of the preparation in the container and the protein content expressed in grams per litre,
- for freeze-dried preparations, the quantity of protein in the container,
- the route of administration,
- for freeze-dried preparations, the name or composition and the volume of the reconstituting liquid to be added,
- where applicable, that the preparation is suitable for use in the prophylaxis of hepatitis A infection,
- where applicable, the anti-hepatitis A virus activity in International Units per millilitre,
- where applicable, the name and amount of antimicrobial preservative in the preparation.

01/2005:0918

HUMAN NORMAL IMMUNOGLOBULIN FOR INTRAVENOUS ADMINISTRATION

Immunoglobulinum humanum normale ad usum intravenosum

DEFINITION

Human normal immunoglobulin for intravenous administration is a liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G (IgG). Other proteins may be present. Human normal immunoglobulin for intravenous administration contains the IgG antibodies of normal subjects. This monograph does not apply to products intentionally prepared to contain fragments or chemically modified IgG.

Human normal immunoglobulin for intravenous administration is obtained from plasma that complies with the requirements of the monograph on *Human plasma for fractionation (0853)*. No antibiotic is added to the plasma used.

PRODUCTION

The method of preparation includes a step or steps that have been shown to remove or to inactivate known agents of infection; if substances are used for inactivation of viruses, it shall have been shown that any residues present in the final product have no adverse effects on the patients treated with the immunoglobulin.

The product shall have been shown, by suitable tests in animals and evaluation during clinical trials, to be well tolerated when administered intravenously.

Human normal immunoglobulin is prepared from pooled material from not fewer than 1000 donors by a method that has been shown to yield a product that:

- does not transmit infection,
- at an immunoglobulin concentration of 50 g/l, contains antibodies for at least 2 of which (one viral and one bacterial) an International Standard or Reference Preparation is available, the concentration of such antibodies being at least 3 times that in the initial pooled material,
- has a defined distribution of immunoglobulin G subclasses,
- complies with the test for Fc function of immunoglobulin (*2.7.9*).

Human normal immunoglobulin for intravenous administration is prepared as a stabilised solution or as a freeze-dried preparation. A stabiliser may be added. In both cases the preparation is passed through a bacteria-retentive filter. The preparation may subsequently be freeze-dried and the containers closed under vacuum or under an inert gas. No antimicrobial preservative is added either during fractionation or at the stage of the final bulk solution.

The stability of the preparation is demonstrated by suitable tests carried out during development studies.

CHARACTERS

The liquid preparation is clear or slightly opalescent and colourless or pale yellow. The freeze-dried preparation is a hygroscopic, white or slightly yellow powder or solid friable mass.

For the freeze-dried preparation, reconstitute as stated on the label immediately before carrying out the identification and the tests, except those for solubility and water.

IDENTIFICATION

Examine by a suitable immunoelectrophoresis technique. Using antiserum to normal human serum, compare normal human serum and the preparation to be examined, both diluted to contain 10 g/l of protein. The main component of the preparation to be examined corresponds to the IgG component of normal human serum. The preparation to be examined may show the presence of small quantities of other plasma proteins; if human albumin has been added as a stabiliser, it may be seen as a major component.

TESTS

Solubility. For the freeze-dried preparation, add the volume of the liquid stated on the label. The preparation dissolves completely within 30 min at 20-25 °C.

pH (*2.2.3*): 4.0 to 7.4.

Dilute the preparation to be examined with a 9 g/l solution of *sodium chloride R* to obtain a solution containing 10 g/l of protein.

Osmolality (*2.2.35*): minimum 240 mosmol/kg.

Total protein. Dilute the preparation to be examined with a 9 g/l solution of *sodium chloride R* to obtain a solution containing about 15 mg of protein in 2 ml. To 2.0 ml of this solution in a round-bottomed centrifuge tube add 2 ml of a 75 g/l solution of *sodium molybdate R* and 2 ml of a mixture of 1 volume of *nitrogen-free sulphuric acid R* and 30 volumes of *water R*. Shake, centrifuge for 5 min, decant the supernatant liquid and allow the inverted tube to drain on filter paper. Determine the nitrogen in the centrifugation residue by the method of sulphuric acid digestion (*2.5.9*) and calculate the content of protein by multiplying the result by 6.25. The preparation contains not less than 30 g/l of protein and not less than 90 per cent and not more than 110 per cent of the quantity of protein stated on the label.

Protein composition. Examine by zone electrophoresis (*2.2.31*), using strips of suitable cellulose acetate gel as the supporting medium and *barbital buffer solution pH 8.6 RI* as the electrolyte solution.

Test solution. Dilute the preparation to be examined with a 9 g/l solution of *sodium chloride R* to an immunoglobulin concentration of 30 g/l.

Reference solution. Reconstitute *human immunoglobulin for electrophoresis BRP* and dilute with a 9 g/l solution of *sodium chloride R* to a protein concentration of 30 g/l.

To a strip apply 4.0 µl of the test solution as a 10 mm band or apply 0.4 µl per millimetre if a narrower strip is used. To another strip apply in the same manner the same volume of the reference solution. Apply a suitable electric field such that the albumin band of normal human serum applied on a control strip migrates at least 30 mm. Stain the strips with *amido black 10B solution R* for 5 min. Decolourise with a mixture of 10 volumes of *glacial acetic acid R* and 90 volumes of *methanol R* so that the background is just free of colour. Develop the transparency of the strips with a mixture of 19 volumes of *glacial acetic acid R* and 81 volumes of *methanol R*. Measure the absorbance of the bands at 600 nm in an instrument having a linear response over the range of measurement. Calculate the result as the mean of 3 measurements of each strip. In the electropherogram obtained with the test solution, not more than 5 per cent of protein has a mobility different from that of the principal band. This limit is not applicable if albumin has been added to the preparation as a stabiliser; for such preparations, a test for protein composition is carried out during manufacture before addition of the stabiliser. The test is not valid unless, in the electropherogram obtained with the reference preparation, the proportion of protein in the principal band is within the limits stated in the leaflet accompanying the reference preparation.

Distribution of molecular size. Liquid chromatography (*2.2.29*).

Test solution. Dilute the preparation to be examined with a 9 g/l solution of *sodium chloride R* to a concentration suitable for the chromatographic system used. A concentration in the range 4 g/l to 12 g/l and injection of 50 µg to 600 µg of protein are usually suitable.

Reference solution. Dilute *human immunoglobulin BRP* with a 9 g/l solution of *sodium chloride R* to the same protein concentration as the test solution.

Column:

- *size: l* = 0.6 m, *Ø* = 7.5 mm,
- *stationary phase: hydrophilic silica gel for chromatography R* of a grade suitable for fractionation of globular proteins with relative molecular masses in the range 10 000 to 500 000.

Mobile phase: dissolve 4.873 g of *disodium hydrogen phosphate dihydrate R*, 1.741 g of *sodium dihydrogen phosphate monohydrate R*, 11.688 g of *sodium chloride R* and 50 mg of *sodium azide R* in 1 litre of *water R*.

Flow rate: 0.5 ml/min.

Detection: spectrophotometer at 280 nm.

In the chromatogram obtained with the reference solution, the principal peak corresponds to IgG monomer and there is a peak corresponding to dimer with a relative retention to the principal peak of about 0.85. Identify the peaks in the chromatogram obtained with the test solution by comparison with the chromatogram obtained with the reference solution; any peak with a retention time shorter than that of dimer corresponds to polymers and aggregates. The preparation to be examined complies with the test if, in the chromatogram obtained with the test solution:

- *relative retention:* for monomer and dimer, the relative retention to the corresponding peak in the chromatogram obtained with the reference solution is 1 ± 0.02;
- *peak area:* the sum of the peak areas of monomer and dimer represent not less than 90 per cent of the total area of the chromatogram and the sum of the peak area of polymers and aggregates represents not more than 3 per cent of the total area of the chromatogram. This requirement does not apply to products where albumin has been added as a stabiliser; for products stabilised with albumin, a test for distribution of molecular size is carried out during manufacture before addition of the stabiliser.

Anticomplementary activity (*2.6.17*). The consumption of complement is not greater than 50 per cent (1 CH_{50} per milligram of immunoglobulin).

Prekallikrein activator (*2.6.15*): maximum 35 IU/ml, calculated with reference to a dilution of the preparation to be examined containing 30 g/l of immunoglobulin.

Anti-A and anti-B haemagglutinins (*2.6.20*). Carry out the tests for anti-A and anti-B haemagglutinins. If the preparation to be examined contains more than 30 g/l of immunoglobulin, dilute to this concentration before preparing the dilutions to be used in the test. The 1:64 dilutions do not show agglutination.

Water. Determined by a suitable method, such as the semi-micro determination of water (*2.5.12*), loss on drying (*2.2.32*) or near infrared spectrophotometry (*2.2.40*), the water content is within the limits approved by the competent authority.

Sterility (*2.6.1*). It complies with the test for sterility.

Pyrogens (*2.6.8*). It complies with the test for pyrogens. Inject per kilogram of the rabbit's mass a volume equivalent to 0.5 g of immunoglobulin but not more than 10 ml per kilogram of body mass.

Antibody to hepatitis B surface antigen: minimum 0.5 IU/g of immunoglobulin, determined by a suitable immunochemical method (*2.7.1*).

STORAGE

For the liquid preparation, store in a colourless glass container, protected from light, at the temperature stated on the label. For the freeze-dried preparation, store in an airtight colourless glass container, protected from light, at a temperature not exceeding 25 °C.

LABELLING

The label states:

- for liquid preparations, the volume of the preparation in the container and the protein content expressed in grams per litre,
- for freeze-dried preparations, the quantity of protein in the container,
- the amount of immunoglobulin in the container,
- the route of administration,
- for freeze-dried preparations, the name or composition and the volume of the reconstituting liquid to be added,
- the distribution of subclasses of immunoglobulin G present in the preparation,
- where applicable, the amount of albumin added as a stabiliser,
- the maximum content of immunoglobulin A.

EXHIBIT 18

THIS EXHIBIT HAS BEEN REDACTED IN ITS ENTIRETY